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VARIABILITY IN PYTHIUM ARRHENOMANES IN RELATION TO ROOT ROT OF SUGARCANE AND CORN [1]

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INTRODUCTION

This paper presents the results of one phase of an investigation of the so-called "root disease" of sugarcane, *Saccharum officinarum* L., in Louisiana. Depletion of the root systems, particularly of ratoon crops, by fungi, favored by a combination of adverse climatic, soil, and biologic factors, is now recognized as a major cause, together with mosaic and red rot, of the widespread failure of D–74 and "native" varieties formerly grown in Louisiana. Despite the general substitution of more resistant canes by the United States Department of Agriculture (*12*),[3] root diseases have continued to have an undetermined but obviously important depressing effect on yields, particularly those on the heavy, poorly drained soils, which comprise about 40 percent of the plantation area. Root rot, caused by *Pythium arrhenomanes* Drechsler, is probably the most important individual component of the complex whenever it assumes importance on the varieties now being grown. The results of studies on the variability of this hitherto poorly defined species, throwing light on questions of taxonomy and physiologic specialization, particularly in relation to distribution and host susceptibility, are presented in this paper.

REVIEW OF LITERATURE

Beginning in 1927, the writers have obtained from decayed roots of sugarcane in Louisiana and other Southern States many hundreds of fungus isolations which have revealed a high proportion of phycomycetous forms belonging mostly to the genus *Pythium*. Of this collection 1 water mold and 13 species of *Pythium* have been identified, including 3 new to science described by Drechsler (*5*, *p. 349*; *6*). Drechsler's further assistance on the morphology has enabled the writers to identify definitely nearly the entire collection. However, identification has been long delayed pending the discovery of a satisfactory culture medium (*11*) for production of the sexual stage so necessary for conclusive identification in most of the species.

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[2] The writers wish to express their thanks to the following members of the staff of the Bureau of Plant Industry: To F. D. Richey and G. F. Sprague for the genetically uniform inbred lines of corn used in these tests; to Eaton M. Summers for valuable assistance in the root-rot survey which yielded many of the cultures used in these comparisons; to Charles Drechsler for guidance in identification of the heterogeneous *Pythium* flora of sugarcane roots; to James E. Taylor and Charles Cassidy for assistance in the greenhouse and laboratory work; and to Edna H. Fawcett for preparation of culture media for the pH studies.

[3] Reference is made by number (italic) to Literature Cited, p. 221.

A preliminary paper (*10*) in 1929 presented evidence showing that the most aggressive sugarcane root-rotting forms should be assigned to *Pythium arrhenomanes*. Approximately 25 percent of the collection may now be placed in this species. However, no particular significance is attached to a definite percentage, because the isolations were not always made during periods most favorable for root rot.

Pythium arrhenomanes was described by Drechsler (*4*) with special reference to a single culture isolated by Helen Johann and considered by Johann, Holbert, and Dickson (*9*) to be the cause of a root rot of corn, *Zea mays* L., in Wisconsin and Illinois. Valleau, Karraker, and Johnson (*15*), in Kentucky, and Branstetter (*2*), in Missouri, have described similar or identical *Pythium* diseases of this crop. Most of these writers emphasize the similarity of their corn root rots to the root rot of sugarcane in Hawaii described in 1921 by Carpenter (*3*). Edgerton (*7*) and his coworkers, who have isolated many *Pythiums* from sugarcane in Louisiana, likewise suggest the probable identity of the more parasitic cane *Pythiums* in the two countries; and in the above-mentioned preliminary paper one of the present writers, by means of culture and pathogenicity tests, showed that the cultures from Louisiana and Hawaii and the type culture from corn should in fact be considered as belonging to the same species. Differences in morphology and behavior in culture were regarded as characters separating strains, or at most physiologic forms, rather than as characters differentiating varieties or species. However, such differences are emphasized by Sideris (*13*), who recently, on the basis of physiologic characters, has described from Hawaii no less than 10 new species or varieties (under the subgeneric name *Nematosporangium*), which the writers have found clearly conform with a somewhat liberal concept of *Pythium arrhenomanes*. All but one of these were isolated from pineapple roots and are stated to be aggressively parasitic on pineapple, sugarcane, corn, and wheat. Vanterpool and Truscott (*16*), of Saskatchewan, have provisionally erected a new variety, *P. arrhenomanes* var. *canadensis*, which proved to be an important causal agent in the browning root rot of cereals.

Considering the many easily recognizable and comparatively constant species of *Pythium*, it is unfortunate that widespread economic importance should be attached to one of such diversity of strains and taxonomic uncertainty. In the comparative studies that follow, an attempt has been made to determine the variability of the species and the extent and constancy of apparent differences between strains [4] as possibly correlated with geographic distribution, host association, and damage. Some of the isolates studied by other workers have been included in the more recent comparisons.

SOURCE OF CULTURES

The abbreviations and the number, locality, host, and origin of the isolates used in this study are as follows:

UNITED STATES SUGARCANE AND CORN ISOLATES

A–14 and E–6.—Washington, D.C., greenhouse corn strains isolated in 1925 by Charles Drechsler.

F series.—Seven Florida cane strains isolated in 1930 from four cane fields on muck and peat soils in the upper peninsular and Everglades districts.

[4] The term "strain" is employed purely for convenience in designating individual isolations ("isolates"), or pure-culture lines derived from single spores or hyphal tips.

I–(2) 132.—Indiana corn strain isolated in August 1928 by Charles Drechsler and J. F. Trost at Lafayette, Ind.

K–20 and K–23.—Kentucky corn strains isolated in February 1926 by Charles Drechsler from diseased corn roots supplied by W. D. Valleau of Lexington, Ky.

L series.—Forty-five representative Louisiana cane strains isolated during 1927–31 from rotting roots of sugarcane collected from the principal soil types throughout the Louisiana sugar district.

Mo.—Missouri corn strain isolated by B. B. Branstetter at Columbia, Mo.

W–27.—Wisconsin corn strain 27, the type culture of *Pythium arrhenomanes* Drechsler, isolated by Helen Johann and studied in relation to corn root rot by Johann, Holbert, and Dickson (*9*).

HAWAIIAN SUGARCANE, PINEAPPLE, AND MISCELLANEOUS ISOLATES

H–2.—Cane strain no. 2 isolated by C. W. Carpenter and received in 1928 from the experiment station of the Hawaiian Sugar Planters' Association.

SN series.—Sideris (*13*), *Nematosporangium* species, type cultures of "Polyandra section" deposited by P. C. Sideris in the type culture collection of the Bureau of Plant Industry, United States Department of Agriculture:

(SN–1) *N. spaniogamon*
(SN–2) *N. arrhenomanes* var. *hawaiiensis*
(SN–3) *N. thysanohyphalon*
(SN–4) *N. hyphalosticton*
(SN–5) *N. polyandron*
(SN–6) *N. rhizophthoron*
(SN–7) *N. leucosticton*
(SN–8) *N. leiohyphon*
(SN–9) *N. epiphanosporon*

CANADIAN CEREAL-BROWNING ROOT-ROT PYTHIUM

Can.—*Pythium arrhenomanes* var. *canadensis* Vanterpool and Truscott (*16*), type culture received August 31, 1932, from T. C. Vanterpool, Saskatoon, Saskatchewan.

MAURITIUS SUGARCANE ROOT-ROT CULTURE

M.—One isolate from the island of Mauritius submitted by S. F. Ashby, of London, to Charles Drechsler for determination.

After much preliminary study and many comparisons of the sexual stage of the above-listed 70 strains or isolates (when a culture medium was finally discovered to produce this stage), it was found that all these cosmopolitan and obviously variant types had enough distinctive characters in common to be classified most usefully under a broadened concept of the species *Pythium arrhenomanes*. At any rate the group is discussed as a whole, and population studies should indicate the relationship and eventual taxonomic disposition of all these related types.

METHOD OF ISOLATION

When present and in an active condition *Pythium arrhenomanes* is easily isolated from flabby root ends or fine rootlets of sugarcane affected by root rot in the southern United States. Aside from usually greater severity of damage, the writers have discovered no specific root symptoms differentiating the attack by this fungus from root-tip rots produced by other species of *Pythium*, particularly by *P. dissotocum* Drechsler and *P. graminicolum* Subr. More abundant cultures have been obtained by making the isolations 2 to 4 days following a rain, especially from small plants during April and May, when soil temperatures are still low enough for rapid spread of infection. During midsummer rootlet-tip rot is common and often severe on susceptible varieties, but the greater activity of secondary invaders, such as *Trichoderma* spp., and of bacterial spreaders makes isolation more difficult.

The root collections may be safely preserved for 1 or 2 days by immediate packing in moist soil in a tightly closed container. In the

laboratory the soil is carefully removed by running water, and the freshly rotted portions of the roots are transferred to Petri dishes containing sterile or ordinary tap water. The water is changed 2 or 3 times during a period of 12 hours, after which the root fragments are pressed dry between disks of filter paper, as recommended by Drechsler, and immediately transferred with flamed forceps to freshly poured plain agar plates. The *Pythiums* usually outgrow the fungal contaminants, permitting their separation. Later the cultures are purified from bacteria by replating them on thick 2-percent plain agar, the agar being inverted into the cover of the plate before the mycelium has reached the boundary, and transfers being made from the original bottom of the disk. The purity of the resultant culture may then be tested by transfers to tubes of neutral bouillon. For experimental study, cultures from a single hyphal tip have been used.

IDENTIFICATION AND SPORULATION

Cultures of *Pythium arrhenomanes* are readily identified in fruiting condition by the usually large number (4 to 20) of crook-necked antheridia mostly of distant origin and disposed in more or less radiate arrangement around the oogonium. Plate cultures viewed under intermediate magnification of the microscope show a characteristic irregularity of mycelium and occurrence of oospores which, in conjunction with the thin walls, dense contents, and radiating antheridial stalks of the latter, present a picture not easily described but so distinctive as unmistakably to differentiate the various strains of this fungus from all other described species of *Pythium*. However, on the usual laboratory culture media sexual fructification among the cane strains of the fungus has been the exception rather than the rule. Those exhibiting any tendency in this direction will usually sporulate in corn-meal agar containing a suspension of grated carrot tissue, as suggested by Johann (*8*), or simply in highly acidified (pH 4.5) corn-meal agar alone, which has been extensively used by the writers. For the nearly 76 percent of the isolates not forming oospores in these media, humic acid-carrot agar, described in a previous publication (*11*), has been successful. Four strains, two from cane and two from corn, remain obstinate, illustrating the physiological differences emphasized by Sideris (*13*). However, on the humic acid-carrot agar all the cultures listed by Sideris have readily fruited in 2 to 6 days.

The four "nonoospore" cane and corn strains are tentatively assigned to *Pythium arrhenomanes* because of their characteristic asexual structure and their parasitism on roots of sugarcane and corn. None of the 13 other species of *Pythium* which have been isolated from sugarcane roots possesses the large distinctive lobulate sporangia characterizing *P. arrhenomanes*. These complex structures are readily formed in a variety of media of which soft-lima-bean agar is perhaps the most satisfactory.

Preliminary comparisons in rate of growth and superficial appearance on a number of vegetable-decoction agars revealed large but often inconsistent physiological differences among the various cultures; for example, differences have been observed in extent of aerial development and waviness of mycelium, formation, and persistence of a matted weft or hyphal disk surmounting the aerial growth in

tube cultures, and in the development of intramatrical, largely abnormal hyphal swellings and mycelial tufts, sporangia, appressoria, and oospores.

POPULATION STUDIES

In order to obtain more accurate quantitative data on the range of variability and degree of possible specialization among the 70 cultures, the oogonia were measured and many of the strains compared in regard to cultural characters and pathogenicity on sugarcane and corn. The population data are summarized in table 1 and are afterward analyzed under appropriate headings. These analyses are followed by comparisons of a limited number that are of particular interest in the root-rot problem in Louisiana.

TABLE 1.—*Comparative population data on 70 isolates of Pythium arrhenomanes from various sources*

Culture designation	Mature oogonia			Mean radial mycelial growth after 48 hours at 35° C. on—		Relative virulence toward inbred lines of corn					
						Lancaster Surecrop				Doctor Yellow Dent (test 46)	
						Test 37		Test 40			
	Number measured	Diameter		Corn-meal agar	Potato agar	Mean dry weight	Reduction from controls	Mean dry weight	Reduction from controls	Mean green weight	Reduction from controls
		Range	Mean								
1	2	3	4	5	6	7	8	9	10	11	12
		μ	*μ*	*Mm*	*Mm*	*Grams*	*Per-cent*	*Grams*	*Per-cent*	*Grams*	*Per-cent*
Controls						1.353		3.14		40.5	
L-11	100	25-44	31	64	39	1.106	18.3	2.97	5.4	32.8	19.0
L-39	50	19-31	24	67	44	1.021	24.5	1.53	51.3		
L-40				70	55	.675	50.1	1.66	47.1		
L-42	50	22-31	26	68	53	.723	46.6	2.18	30.6		
L-50	50	23-33	26	74	50	.734	45.8	2.04	35.0	23.8	41.2
L-58	100	17-28	23	71	51	.936	30.8	1.95	37.9	23.1	43.0
L-61	34	20-30	24	71	43			2.15	31.5		
L-233	54	19-33	27	62	48	1.018	24.8	2.97	5.4	34.6	14.6
L-236-1	53	25-36	30	66	48	1.067	21.1	2.69	14.3		
L-262	61	25-42	32	63	52	.999	26.2	1.01	67.8	22.0	45.7
L-273	46	22-39	29	70	51	1.062	21.5			20.3	49.9
L-318	50	28-44	34	61	48	.550	59.3	1.25	60.2	16.2	60.0
L-319	51	17-28	23	72	55	.763	43.6	2.07	34.1	27.6	31.8
L-348	50	28-39	33	66	46	.996	26.4				
L-350	100	22-42	31	70	41	.905	33.1	1.54	51.0		
L-352	50	25-39	30	65	39	1.077	20.4			27.9	31.1
L-354	67	23-47	35	60	55			1.88	40.1		
L-356				66	41	1.081	20.1	1.37	56.4		
L-379	28	23-37	29	66	48	1.074	20.6			25.6	36.8
L-380	50	19-31	24	69	43	.973	28.1	1.75	44.3		
L-382	49	19-33	26	69	41	.874	35.4	1.89	39.8	23.5	42.0
L-730	48	25-44	33	67	56	1.181	12.7	2.84	9.6	27.1	33.1
L-780	52	19-28	23	66	51	.614	54.6	1.48	52.9	19.2	52.6
L-797	45	23-33	32	65	44	.989	26.9	1.61	48.7	32.1	20.7
L-803	43	23-37	29	65	52	1.094	19.1	2.55	18.8	26.2	35.3
L-804	46	19-31	23	65	43	.999	26.2	1.84	41.4	25.8	36.3
L-806	50	19-33	26	74	42	.879	35.0	1.76	43.9		
L-815	64	22-42	28	71	47	.690	49.0	2.32	26.1	25.1	38.0
L-816	48	20-33	27	75	48	.950	29.8				
L-1103	64	19-31	25	67	48			1.84	41.4	23.8	41.2
L-1141	50	17-25	21	71	56			1.79	43.0	24.1	40.5
L-1143	65	19-33	26	74	54	.865	36.1	1.33	57.6		
L-1144	54	17-28	23	73	55	1.052	22.2			21.7	46.4
L-1147	52	17-28	22	69	53	.754	44.3	1.61	48.7		
L-1278	51	17-30	24	66	45	.714	47.2	1.63	48.1	27.4	32.3

TABLE 1.—*Comparative population data on 70 isolates of Pythium arrhenomanes from various sources*—Continued

Culture designation	Mature oogonia			Mean radial mycelial growth after 48 hours at 35° C. on—		Relative virulence toward inbred lines of corn					
						Lancaster Surecrop				Doctor Yellow Dent (test 46)	
						Test 37		Test 40			
	Number measured	Diameter		Corn-meal agar	Potato agar	Mean dry weight	Reduction from controls	Mean dry weight	Reduction from controls	Mean green weight	Reduction from controls
		Range	Mean								
1	2	3	4	5	6	7	8	9	10	11	12
		μ	μ	*Mm*	*Mm*	*Grams*	*Per-cent*	*Grams*	*Per-cent*	*Grams*	*Per-cent*
L–1279	50	19–31	24	72	52	1.116	17.5	1.18	62.4		
L–1281	56	20–30	27	69	44	.883	34.7	1.83	41.7		
L–1284	50	22–31	25	63	57	.868	35.8	1.45	53.8		
L–1285	50	19–28	24	68	45	.749	44.6	1.63	48.1		
L–1295	48	19–31	23	66	48	1.068	21.1	1.13	64.0		
L–1296	102	19–31	24	73	40	.785	42.0	1.27	59.6		
L–1320	60	19–31	25	68	44	.759	43.9	1.96	37.6		
L–1323	57	19–31	24	62	46	.514	62.0	1.57	50.0	25.1	38.0
L–1324	52	19–31	25	73	45			2.03	35.4		
F–1	50	19–31	24	71	51	1.017	24.8	1.34	57.3		
F–6	69	19–28	22	71	46	1.073	20.7	1.76	43.9	26.2	35.3
F–7	51	22–36	26	73	48	.715	47.2	1.78	43.3		
F–10	52	22–33	26	67	50	1.018	24.8	1.54	51.0		
F–13	55	19–28	24	65	45	.883	34.7	1.65	47.5		
F–21	53	22–31	26	69	38	1.138	15.9	1.38	56.1		
F–23	37	20–30	25	71	34	.709	47.6	1.35	57.0	22.5	44.4
A–14				56	42	.696	48.6	1.35	57.0	23.0	43.2
E–6	10	30–37	33	52	37	.624	53.9	1.49	52.5	23.7	41.5
I–(2)132				45	33	.434	67.9	1.17	62.7	13.6	66.4
K–20	48	25–36	29	35	20	1.333	1.5	2.90	7.6	28.7	29.1
K–23	58	31–50	39	61	51	.952	29.6	2.99	4.8	25.1	38.0
Mo	30	31–50	39	47	20	.952	29.6	2.95	6.1	26.4	34.8
W–27	100	28–44	35	53	31	.800	40.9	2.82	10.2	27.1	33.1
H–2	57	22–33	26	71	46						
M	150	33–56	42								
Can	50	28–44	35								
SN–1	48	23–33	29								
SN–2	49	23–37	30								
SN–3	53	27–50	36								
SN–4	71	30–47	37								
SN–5	56	27–47	39								
SN–6	51	27–37	31								
SN–7	48	27–50	37								
SN–8	40	30–53	38								
SN–9	33	23–50	35								
Combined mean (66 isolates)			28.5								

SIZE OF OOGONIA AND MISCELLANEOUS MORPHOLOGICAL COMPARISONS

The data in columns 2 to 4 of table 1 show the number, range, and mean diameter of mature oogonia for 66 of the isolates grown in aliquots of the same original lot of humic acid-carrot agar. The humic acid A used for this purpose was extracted from a local woodland soil and the culture medium was prepared as specified in a previous publication (*11*). The different isolates were plated (some as many as three times) and the oogonia measured after 2 to 6 days over a period of 6 months in 1932.

Oogonia instead of oospores were measured because of larger numbers and consequently greater freedom and reliability in random

sampling. Even in this medium large numbers of oogonia sometimes failed to develop normal oospores unless special precautions, not known for all the strains, were taken. An oogonium was judged to be mature when a number of antheridia were attached, some of which had begun to clear, indicating fertilization. This was readily determined under moderately low magnification (360 diameters), secured by reflecting the image to table level by the aid of a camera lucida. By this procedure an entire plate could be quickly sampled, whereas dependence upon oospores would necessitate sectioning the agar and using high magnification in order to see the outer wall and also to avoid measuring degenerate spores.

In figure 1 the isolates are arranged in frequency classes of 1-micron grades, according to the mean diameter of oogonia (column 4, table 1), usually based on measurements from a single Petri dish; while the extreme range of diameters of individual oogonia in the entire population of strains was from 17μ to 56μ, the greatest range for a single isolate (SN–9) was 23μ to 50μ. The coefficient of variability of oogonia within a single Petri dish for five isolates showed, as might be expected, relatively low figures, ranging from 10.1 percent for Mauritius to 12.3 percent for L–11. The isolate means ranged from 21μ for L–1141 to 42μ for the Mauritius culture. This range contrasts with 24μ to 35μ given by Drechsler (*4*) in his original description of the species, although the general mean of 28.5μ for 66 isolates in table 1 is about the same as his average of 29.4μ based on the type culture, W–27. On the humic acid-carrot medium this isolate (W–27) is slightly larger than Drechsler's figures. This fact confirms observations and comparative measurements of several other isolates by showing that the latter medium produces not only slightly larger oogonia but more luxuriant mycelial development than the carrot-corn meal agar alone.

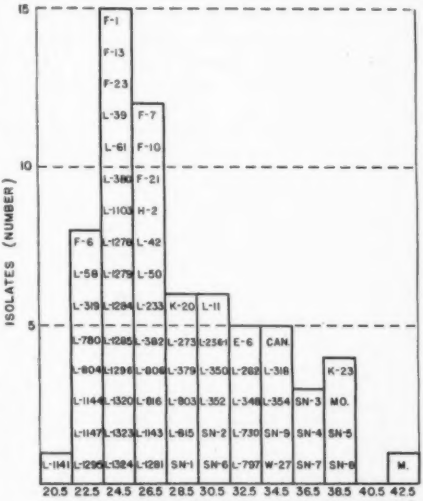

FIGURE 1.—Frequency distribution of 66 isolates of *Pythium arrhenomanes* according to mean diameter of oogonia, produced in humic acid-carrot agar.

In order to determine whether natural humic acids from other sources would be as favorable as A, as well as to secure some measure of reliability for judging the significance of differences in isolate means, shown in figure 1, further measurements, summarized in table 2, were made. Data on 10 isolates are given in table 2, but not all were used in every test. The tests extended over a period of 6 months during 1932, with consequent variation in temperature and other conditions incident to replating. In test 1, carrot-corn meal agar alone was used, and in the remaining tests humic acid at the rate of 50 p.p.m. was

added to this. Humic acid B was extracted from a rich garden soil, and C from decomposed filter-press cake, a 50-percent organic residue from clarification of sugarcane juice. The three isolates plated on B and C, despite presumably widely different humus extracts, fruited as readily and abundantly as on A, and the average oogonial size on these different media is even less variable than those representing successive platings on the same medium. The few that fruited on carrot-corn meal agar alone show, as above stated, slightly smaller oogonia (in 3 out of 4 cases). It seems reasonable to conclude that these results may be fairly accurately compared with measurements of oogonia produced elsewhere by the same or other isolates by the aid of carrot tissue with or without humic acids from different sources.

TABLE 2.—*Influence of culture medium and periodic replatings on mean diameter of oogonia of each of 10 isolates of Pythium arrhenomanes* [1]

[Individual Petri dish means based on diameters of about 50 oogonia]

Isolate no.	Mean diameter of oogonia on—							Mean [1]	Variance [2] (σ^2)
	Carrot agar alone	Humic acid-carrot agar							
		A				B	C		
	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7		
	μ	μ	μ	μ	μ	μ	μ	μ	μ
L-11		29.8	31.8	32.3		32.1	32.1	31.6	1.06
L-58		23.6	21.9	23.9				23.0	1.19
L-318	34.0	34.1						34.0	.01
L-350		30.1	31.9	33.1	33.1	32.2	31.1	31.9	1.37
L-730	31.8	32.8						32.3	.50
L-1296		23.7	24.6					24.2	.41
H-2	26.4	26.3						26.4	.01
K-20	28.0	29.2						28.6	.72
M		42.3	41.2	40.3	40.8	41.9	42.1	41.4	.63
W-27		35.6	34.2			35.3		35.0	.55
Weighted mean variance									[3] .78

[1] Standard error of single isolate mean ($\sqrt{0.78}$) = 0.88μ.

Standard error of difference between single isolate means ($\sqrt{2} \times 0.88$) = 1.25μ.

Difference between individual Petri dish means required for significance (odds ±1,000:1) = 1.25 × 3 = 3.75 (about 4μ).

[2] Variance reached for each isolate by dividing the sum of the squared deviations from the isolate mean by ($n-1$).

[3] The variance for each isolate is weighted by the number of tests, or successive Petri dish means, from which it was determined.

The differences among the series of means for a given individual isolate in table 2 indicate the extent of variability due to accidental or mainly nongenetic factors. Since the 10 isolates in table 2 represent the entire size range of the population of isolates in table 1 and figure 1, their average variance, weighted according to the number of tests and converted to the standard error of the difference, gives some indication as to how much of the apparent difference between isolates in table 1 may be due to chance. If this assumption is justified, it may be seen from table 2 that a difference exceeding 4μ between frequency classes in figure 1 may be considered a result of inherent difference between the respective groups of isolates.

Figure 1 suggests strongly the probable existence in nature of a considerable number of morphologic strains or lines which make up the collective or natural species *Pythium arrhenomanes*. Despite the uniformity and consistency of oogonial size in individual isolates or in groups and the wide and significant differences between certain groups, there are enough intermediate or overlapping classes to resolve

the entire collection into a single integrating natural unit. After all, the differences are quantitative rather than qualitative, the preliminary studies of other features showing no important and consistent morphologic presence-absence characters which might be correlated with spore size or otherwise employed for distinguishing isolates. For example, the number and disposition of antheridia are exceedingly variable, although in general the larger oogonia of the same or of different isolates may have the greater number of antheridia.

The Louisiana and Florida cultures fall mainly in the smaller spore range, whereas the Sideris isolates and most of the corn and miscellaneous representatives occupy the larger oogonial classes, suggesting some regional differences. Whether greater regional representation, particularly from the areas growing maize or other cereals and from Hawaii and Mauritius, would correct the pronounced skewness of figure 1 or develop a bimodal distribution (indicating morphologic varieties) cannot, of course, be predicted from present data. In the latter event figure 1 shows that there would still be enough overlapping, at least in spore size, to limit the general usefulness of such a separation. Figure 2 shows outline tracings of oogonia representing several of the size classes as well as other morphologic features already enumerated that are considered of greater importance in showing conspecific relationship.

The Sideris *Nematosporangium* "species" here included show as a group a narrower range of oogonial diameter than do the Louisiana cultures, possibly because of the smaller population of cultures represented. Sideris (*13*) states that morphologic differences between his species are "almost insignificant, rarely exceeding those of normal variation". Figure 1 shows that in the humic acid agar his numbers 1, 2, and 6 have significantly smaller oogonia than any of the others. Beyond this no detailed comparative studies have been made. It is not clear from his publication whether Sideris erected these 6 new species and 1 new variety from pineapple and the 1 species from *Billbergia* on the basis of studies of a single isolate of each or on populations of similarly behaving isolates all the representatives of which could be differentiated from the remaining "species" populations by the characters given in his key. In either event the separation of new species on relatively minor differences is of questionable value. Recognizing the basic fact that no two living things in nature are apt to be exactly alike in all respects, the consistent smaller differences between groups, particularly of a functional character, might be recognized, if necessary, by the designation of physiologic forms. The writers believe that in the genus *Pythium* the concept of the species and even of the variety should be based on well-defined morphologic characters and should include the usual range of variability common to all groups of closely related organisms in nature.

The variability of *Pythium arrhenomanes* is recognized by Vanterpool and Truscott (*16*), who also have compared a series of isolates from different sources and have refrained from naming each variant a new species. Oogonial measurements and collateral studies of one representative of their new variety *canadensis* indicate its morphologic identity with a number of isolates included in figure 1. Since most of the physiological comparisons that follow were made prior to the publications of Vanterpool and Truscott and of Sideris, their cultures

could not be included and discussed herein in relation to the writers' collection. However, in laboratory and culture-chamber inoculation

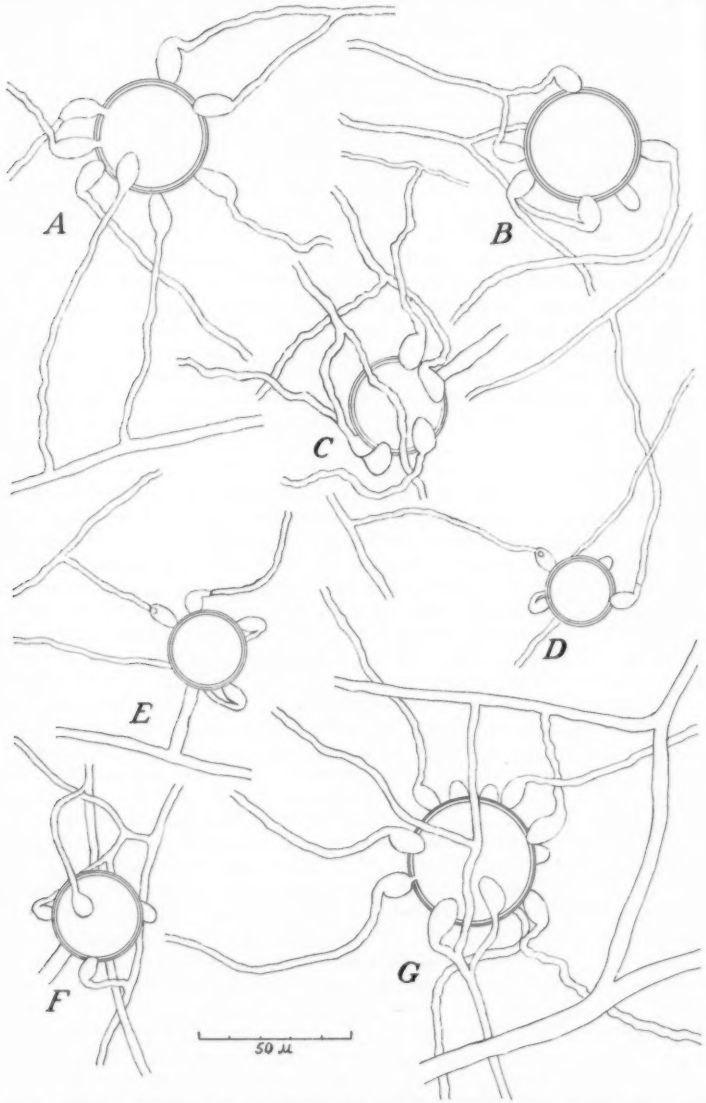

FIGURE 2.—Size of oogonia and number of antheridia representative of different isolates of *Pythium arrhe nomanes*, formed in humic acid-carrot-corn meal agar plates: *A*, L-11; *B*, W-27 (type culture); *C*, H-2 *D*, L-58; *E*, L-1143; *F*, L-350; and *G*, Mauritius. Outline tracings by aid of a camera lucida. × 460.

tests, all their cultures proved to be pathogenic to sugarcane (Louisiana Purple variety).

RELATION TO TEMPERATURE IN CULTURE

Preliminary tests in 1928 and 1929 with a series of the Louisiana cultures on various vegetable-decoction agars showed no growth after 6 days at 2° C. but perceptible growth over the range 6° to 36°. These tests were conducted mainly to study the influence of temperature on sporulation; but, as already indicated, most of the cultures failed to fruit at any temperature and those that did were too variable and undependable on repetition of the tests to make sporulation of value for possible classification purposes. However, wide differences were noted in rapidity of mycelial growth, and at the highest temperature the one corn isolate (W–27) used as a control noticeably lagged behind all others. Accordingly, population comparisons were conducted at 35°.

STUDIES ON CORN-MEAL AND POTATO AGARS AT 35° C.

Columns 5 and 6 of table 1 give the radial growth for each of 59 of the isolates, expressed as the mean of 10 plates per isolate, for two successive tests, one on white corn-meal agar (pH 6.2) and the other on Thaxter's potato agar (pH 5.6). The 590 Petri dishes required for each experiment were inoculated at one side from fresh cultures on the same lot of medium, and after perceptible growth the margin of advancing mycelium was marked with india ink and the cultures carefully arranged for uniform exposure in a large water-bath incubator. After 48 hours at 35° C. the margin on the same radial axis was again marked and the extent of growth measured and tabulated.

The data in columns 5 and 6 (table 1) are arranged in frequency classes in figure 3. Corn-meal agar is obviously much more favorable for growth than potato agar. The slower growth, irregularity of hyphal disks, and variability of replicates on the latter make the results less reliable, although from the preliminary studies one could not expect every isolate to hold necessarily the same relative position on different nutrients. The classification of growth on corn-meal agar separates the isolates (with but 2 exceptions) into 2 groups according to host and geographic origin, the subtropical sugarcane strains from Florida, Hawaii, and Louisiana showing as a group greater adaptability and uniformity of reaction to high temperature than the corn isolates. The combined average of the cane isolates is 26.9 percent greater on corn-meal agar and 29.5 percent greater on potato agar than the respective means for the corn group. Therefore, physiologic specialization in response to temperature appears to be indicated. However, as in oogonial size, there are enough intermediate representatives of both groups to limit the general usefulness of any subspecies classification based on such criteria.

COMPARISON OF SELECTED CANE AND CORN ISOLATES OVER A RANGE OF TEMPERATURES

In order to determine the optimum temperature for growth in culture media and the magnitude of possible differences between strains over a range of temperatures, two experiments were carried out. For this work the Ayers, Rupp, and Johnson synthetic agar medium, as modified in the Manual of Methods for Pure Culture Study of Bacteria (*14, p. B11*), was employed.

Experiment A (fig. 4) was initiated before the acidity studies reported herein had been completed, so that the hydrogen-ion concen-

tration of the synthetic medium was adjusted empirically to near neutrality (pH 6.6), whereas that in experiment B (fig. 5), conducted later, was adjusted to pH 5.9. In experiment A, 1 percent of dextrose was added before sterilization, which caused no significant shift in reaction of the nearly neutral medium. In experiment B, 1 cc of a separately autoclaved 10-percent solution of dextrose was added after sterilization to each 10-cc tube of melted agar. The technic of inoc-

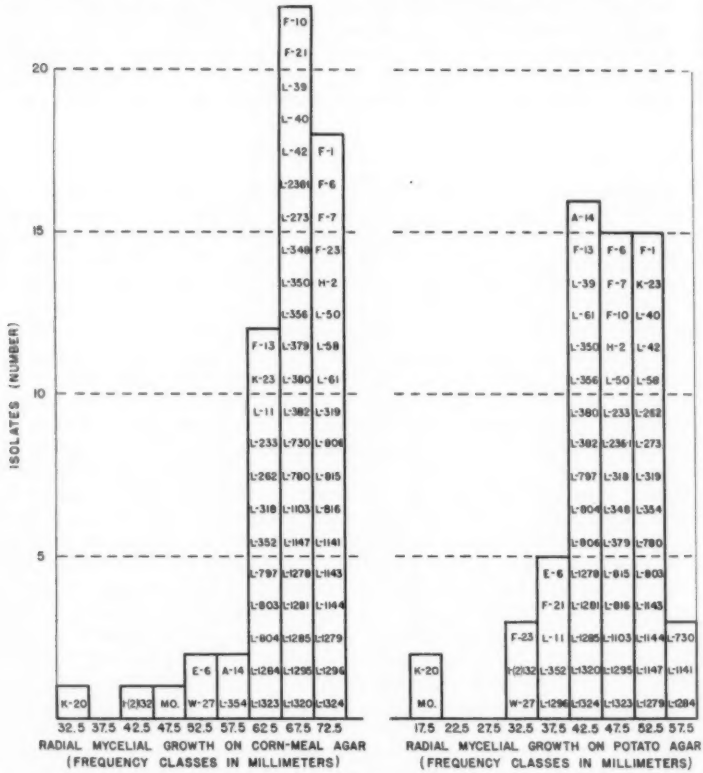

FIGURE 3.—Frequency distribution of 59 isolates of *Pythium arrhenomanes* according to the extent of radial mycelial growth on 2 culture media after 48 hours at 35° C.

ulation, preincubation, and growth measurement after 48 hours was identical with that employed in the preceding population study on corn-meal and potato agar, except that the means in figures 4 and 5 are based on 4 instead of 10 replications.

The curves in figures 4 and 5 indicate that on this synthetic medium 30° C. is near the optimum temperature for most of the strains except the Indiana culture, which shows a decisive optimum at 25°. At 42° all the cultures died within 48 hours. The nice segregation of isolates in figure 4, according to respective hosts, becomes obscure in figure 5, with the more acid medium favoring some of the corn isolates, and the

inclusion of additional cane representatives, particularly F–13, which occupies an intermediate position. Nevertheless, at 35° the differentiation is still clear-cut and confirms the results of the preceding larger test (fig. 3) in showing the greater adaptation or tolerance of

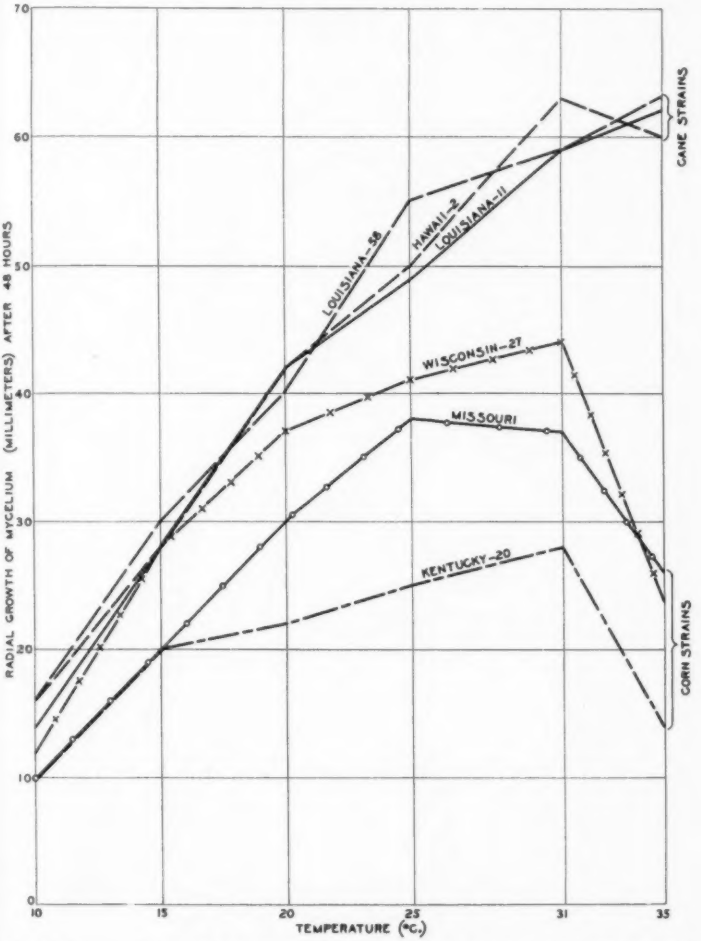

FIGURE 4.—Comparative mycelial growth after 48 hours over a range of temperatures (10° to 35° C.) of 3 isolates of *Pythium arrhenomanes* from sugarcane and 3 from corn, grown in synthetic agar at pH 6.6 (temperature experiment A).

the cane strains to high temperatures. This would seem quite natural in view of the more southerly origin of the latter.

RELATION OF pH OF CULTURE MEDIUM TO GROWTH

Possible differential response of the various strains to the hydrogen-ion concentration of the nutrient medium, suggested by the preceding

temperature studies, was tested in preliminary experiments by varying the pH values of the same synthetic agar. This was accomplished by adjusting the proportions of monophosphates and diphosphates of the medium to give the desired reaction. The methods

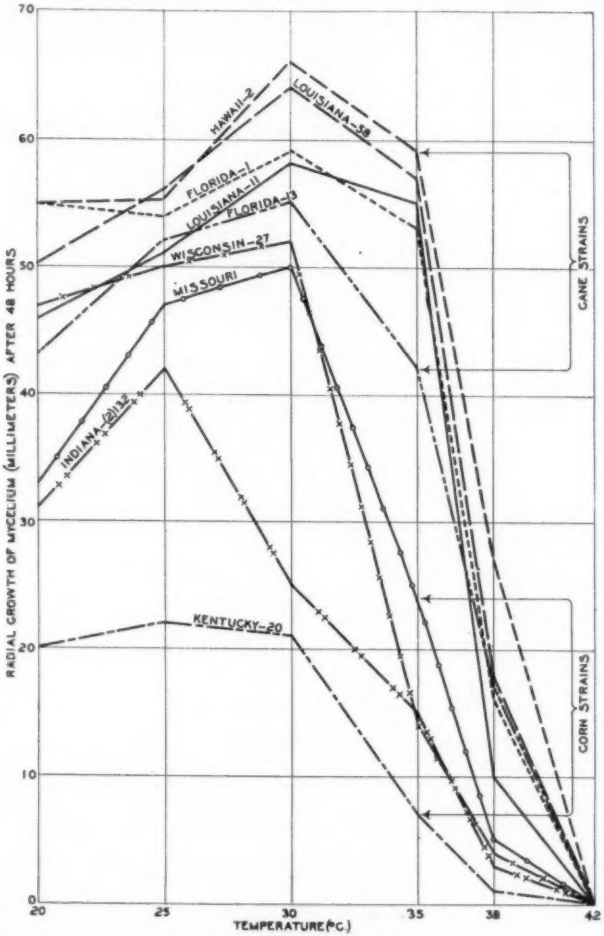

FIGURE 5.—Comparative mycelial growth after 48 hours over a range of temperature (20° to 42° C.) of 5 isolates of *Pythium arrhenomanes* from sugarcane and 4 from corn, grown in synthetic agar at pH 5.9 (temperature experiment B).

employed were the same as in the preceding experiment B. Five plates of each pH value were inoculated with each of the five strains. The cultures were immediately transferred to an incubator at 31° C., and after a few hours the advancing margin of renewed growth was marked on the bottom of the plate with india ink. Radial growth beyond the mark was measured after 48 hours.

The experiment was repeated 3 times from aliquots of the original batch of medium, but after the first test a shift of pH in the alkaline range, resulting in more rapid growth, made it impossible to

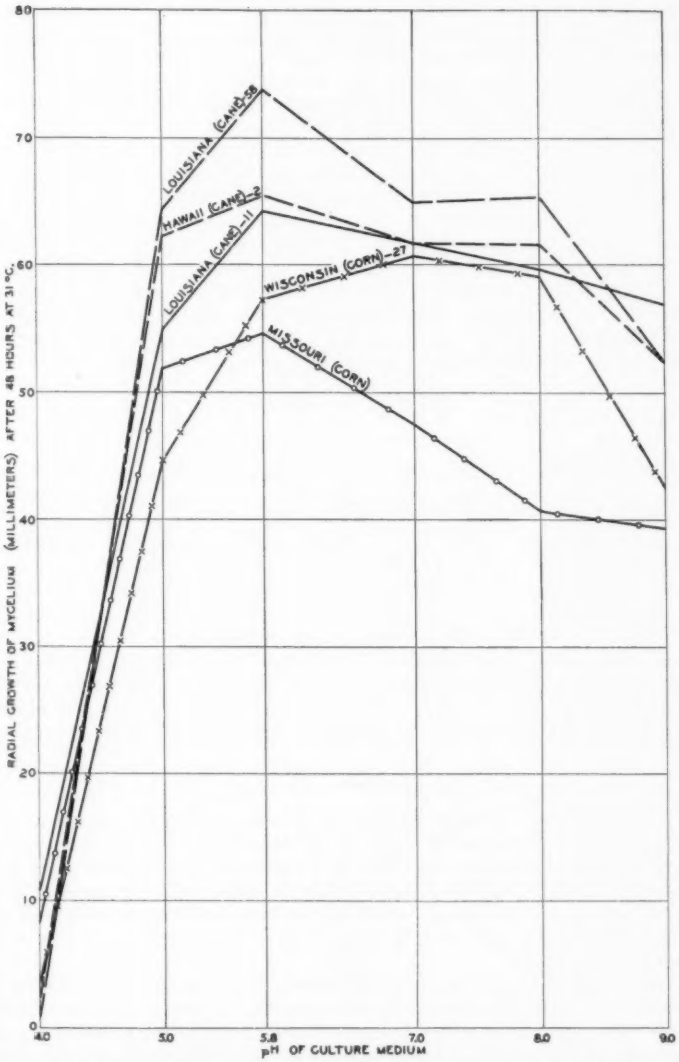

FIGURE 6.—Differential response of five isolates of *Pythium arrhenomanes* to reaction of culture media.

obtain a reliable average for the 3 series. For example, after 10 days the original pH 7 had changed to pH 6.9, 8 to 7.7, and 9 to 8.6. Therefore, the results of only the first series are presented in figure 6.

The curves in figure 6 show wide and statistically significant differences in growth rate among most of the strains at a given pH value of the substratum. Two of the curves show some evidence of an isoelectric point near pH 7. As judged by the shape of the curve, the Wisconsin (type culture) is apparently the most divergent strain. Its optimum tends toward the alkaline side, whereas the Missouri culture apparently favors the more acid range (pH 5 to 5.8). The three cane strains made most rapid growth at pH 5.8 and their curves in general are in closer agreement than the corn cultures when compared both individually and collectively.

Two or three of the pH 4 plates of each strain were placed in the ice box (12° to 15° C.) and after 2 weeks again compared. The Missouri culture was most advanced, having completely covered the plates; L–58 had nearly reached the margin, while H–2, L–11, and W–27 occupied approximately two-thirds of the plate area. L–11, W–27, and to a lesser extent H–2 showed abnormal mycelial types at this high acidity, the hyphae being knotted and very irregular in branching and growth. The Missouri and L–58 cultures were normal in this respect, which supports the evidence in figure 6 that the Missouri culture particularly is a more acid-tolerant form.

PATHOGENIC RELATIONS

Comparisons on Inbred Corn

The divergent behavior of isolates as individuals or as groups, shown in the preceding physiological experiments, emphasized the importance of possible differences in pathogenic relations, especially if such differences might be correlated with geographic distribution of the strains or varieties of the host. Relative virulence as a criterion of physiologic specialization, as well as of potential crop damage, has been studied intensively for a few of the cane strains, but inadequately from the broader standpoint of species population. Reliable comparison of a large number of isolates in the limited greenhouse space has necessitated dependence mainly upon corn in preference to sugarcane as the test host. The results of three inoculation experiments (nos. 37, 40, and 46) on corn are presented in table 1, columns 7 to 12.

EXPERIMENTAL METHODS

For the purpose of securing more or less reproducible results, quartz sand watered with a satisfactory nutrient solution was used for growing the plants, and the experiments were terminated after a growth period of 5 to 8 weeks. Limitations of space and equipment prohibited carrying the plants to a larger size, which naturally might have given results different in relative as well as absolute value from those here obtained.

The plants were grown in 5-inch clay pots coated with asphaltum and filled with a high-grade quartz sand. Three times weekly the plants were given a nutrient solution containing the following salts in the partial volume-molecular concentrations indicated: Ammonium nitrate (NH_4NO_3), 0.006; potassium dihydrogen phosphate (KH_2PO_4), 0.0015; potassium monohydrogen phosphate (K_2HPO_4), 0.0005; magnesium sulphate ($MgSO_4$), 0.001; calcium chloride ($CaCl_2$), 0.003; plus rarer elements in parts per million: Iron (Fe) (as ferric

citrate), 5; manganese (Mn), 5; boron (B), 0.53; copper (Cu), 0.25; aluminum (Al), 0.56; zinc (Zn), 0.25; and bromine (Br), 0.26. The hydrogen-ion concentration of the dilute nutrient was pH 6.2.

The corn seeds were surface-disinfected by 10 minutes' submersion in a solution of 1:1,000 mercuric chloride ($HgCl_2$) in 50-percent alcohol, rinsed in three changes of boiled water, and soaked overnight; they were then either sprouted between blotters or planted directly 1 to 3 grains per pot and subsequently thinned according to uniformity of size to a single plant per pot. Each pot was inoculated with four small agar squares from a 2-day-old corn-meal agar Petri-dish culture of the isolate to be tested, and similar but sterile agar squares were added to the controls. In experiment 37 the pots were inoculated at the time of planting and in experiments 40 and 46 the pots were inoculated when the plants were 3 to 4 inches tall. Thus, in the latter experiments the complicating effect of germination injury, admittedly important under field conditions, was obviously eliminated as a source of variability. To equalize differences due to position, the various replicates of both inoculated and control cultures were distributed at random on the benches. Later, as the plants grew larger, distilled water was added daily, with an occasional complete flushing to prevent undue concentration of nutrient salts. The pH of leachings showed an extreme variation (usually on the acid side) never exceeding one full unit.

EXPERIMENTS 37 AND 40

An F_8 inbred pedigreed selection (no. 608–325) of the Lancaster Surecrop variety of corn was used in experiment 37 and the next, or F_9, of the same selection was used in experiment 40. In the former there were 10 replications per isolate and 20 controls, and in the latter 5 replications and 10 controls.

Experiment 37, extending from May 20 to June 27, 1932, was conducted in an open coldframe outdoors, where the temperature of the sand ranged from 17° to 28° C., being about 18° for a large portion of the period; experiment 40, extending from January 9 to February 20, 1933, was confined to the greenhouse. The uniformly higher and more constant sand temperature (about 21°) of the latter experiment gave, despite poorer illumination, generally greater dry weights per unit of time than experiment 37. Figure 7 shows typical differences in size of plants in experiment 40 resulting from inoculation with mildly, intermediately, or virulently parasitic strains isolated from corn (*A*) and from sugarcane (*B*). Yield data are presented in columns 7 to 10 of table 1 and later statistically analyzed in conjunction with those from experiment 46.

EXPERIMENT 46

In order to determine whether similar relative pathogenicity would be manifested toward a different variety of corn, a smaller but selected group of the isolates was tested on an eighth-year selfed line of the Doctor Yellow Dent variety from Iowa. In this experiment previously sprouted seed was planted November 1, 1933, in the greenhouse. Otherwise the experiment was carried out as were earlier tests. Inoculum was added December 13, when the plants were about 4 inches tall, 10 replications per isolate and 10 control pots being used;

the plants were harvested January 3, 1934. Sand temperature in the center of the pot ranged from 16° to 28° C., but for most of the period the daily mean fluctuated within a few degrees of 21°. Therefore, experimental procedure and environmental conditions were similar to those of experiment 40.

At the termination of the experiment the uninoculated or control plants were 12 to 15 inches tall, and the inoculated plants showed much the same range of stunting by the various isolates as had been observed in previous tests. The plants were cut off just above the

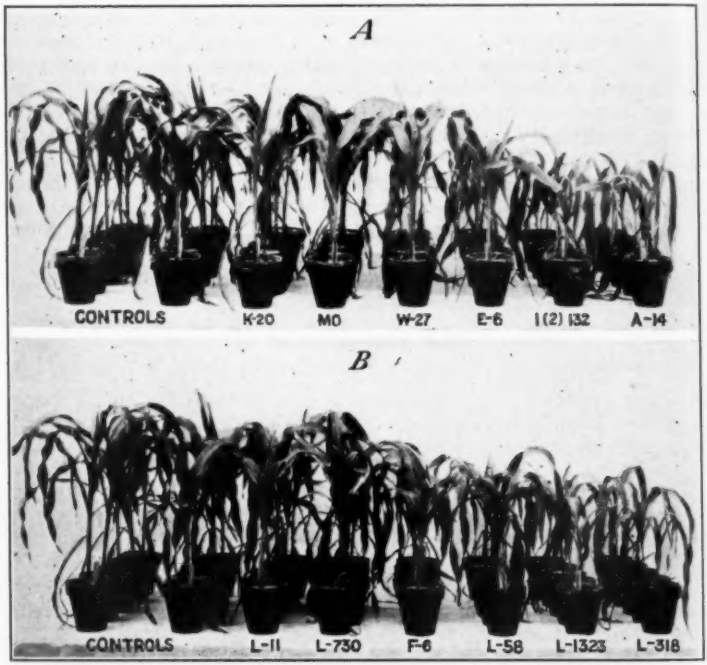

FIGURE 7.—Relative virulence of a series of strains of *Pythium arrhenomanes* isolated from corn (*A*) and from sugarcane (*B*) to an F_9 inbred selection of the Lancaster Surecrop variety of corn (experiment 40).

highest brace roots and weighed at once. Then the roots were washed free from the sand and the extent of injury was noted. While the roots were similar in appearance to those in previous tests, the extent of injury here varied considerably, within as well as between series, despite uniform treatment and favorable moisture and temperature for infection. Possibly the usual but smaller variations in infection obtained on the line of Lancaster Surecrop were here accentuated by greater genetic variability toward infection by another inbred line of corn. Figure 8 contrasts typical root development of the controls with that of plants exposed to virulently or mildly parasitic cane strains of the fungus. Infection by the latter (also typical of earlier results) was confined to the smallest rootlets and was more noticeable either at the bottom of the pot, where the

moisture content of the sand was highest, or near the sand surface, where possible weakening of rootlets by moisture fluctuations and occasional drying occurred. The virulent strains not only destroyed the rootlets but prevented elongation of a majority of the main roots.

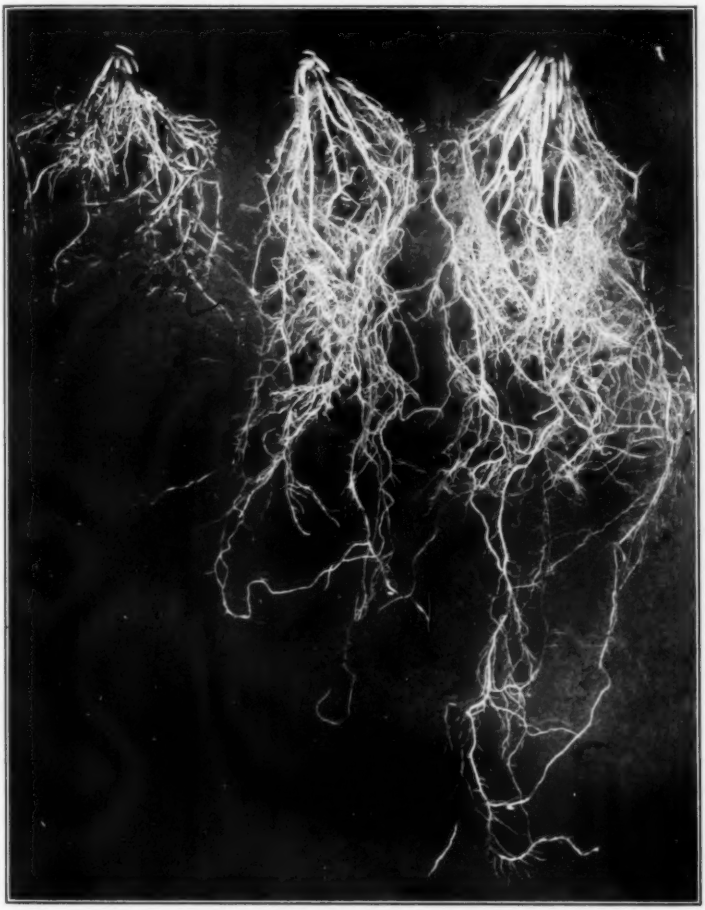

FIGURE 8.—Comparative root-rot damage in experiment 46 of an F_8 selfed line of the Doctor Yellow Dent variety of corn by (*A*) virulently and (*B*) mildly parasitic cane strains of *Pythium arrhenomanes*, contrasted with a control (uninoculated) plant (*C*). Photographed by James F. Brewer.

Yield data are presented in columns 11 and 12 of table 1 and are then statistically analyzed and discussed in conjunction with those from experiments 37 and 40.

STATISTICAL ANALYSES OF YIELD DATA FROM EXPERIMENTS 37, 40, AND 46

The variability of weights of tops among individual replicates from which the mean values in table 1 were derived has been statistically

analyzed by the variance method, and the generalized standard error and required difference between means for significance have been calculated for each experiment, as shown in table 3. When the difference in number of grams between means required for significance is expressed as a percentage of the controls, the reliability of the percentage reduction by each isolate (columns 8, 10, and 12, table 1) and differences between isolates may be judged. Accordingly, the 0.24 g required in experiment 37 is found to be 17.7 percent of 1.353, the mean yield of controls, so that the actual percentage reduction by any isolate given in column 8 of table 1 must at least equal this figure to indicate that the reduction was not due to chance alone. Corresponding required values for tests 40 and 46 are 22.9 percent and 18.4 percent, respectively.

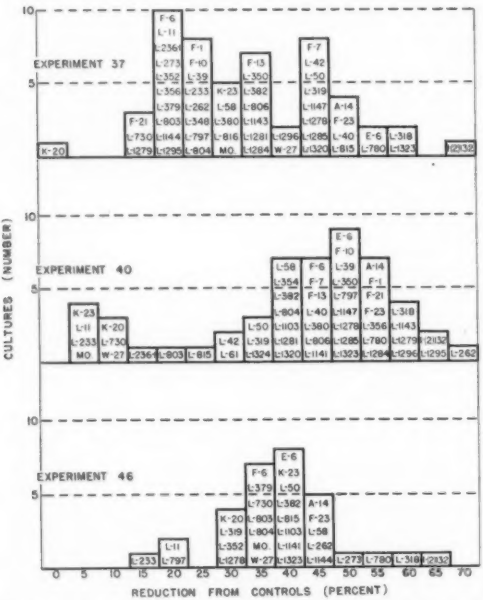
EXPERIMENT 37
EXPERIMENT 40
EXPERIMENT 46
CULTURES (NUMBER)
REDUCTION FROM CONTROLS (PERCENT)
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70

FIGURE 9.—Frequency distribution of a series of cultures of *Pythium arrhenomanes* according to the percentage reduction in weight of tops (compared with controls) from inoculation of two inbred lines of corn (experiments 37 and 40 on the Lancaster Surecrop, and experiment 46 on the Doctor Yellow Dent variety; data from table 1, columns 8, 10, and 12).

In order to facilitate comparison, the percentages in table 1 are thrown into frequency classes in figure 9, and those isolates falling within each 5-percent class are so designated. Unfortunately, greenhouse space and facilities prohibited including all isolates in every test. Nevertheless, enough of the population was tested in each case to warrant at least preliminary conclusions regarding strain differences on the same and different varieties.

CONCLUSIONS

The high z values in table 3, greatly exceeding the 1-percent point, indicate that a high degree of significance may be attached to differences between fungus strains. This is true in spite of great variability between individual inoculated plants, particularly in experiment 46. However, the individual variability in all tests has given such large experimental errors that the percentage reduction by several of the mildly parasitic isolates (fig. 9), when compared with the abovementioned percentage equivalent of the error, is of dubious significance. A larger number of replications would of course be necessary to obtain more reliable values for the reductions by these weakly parasitic forms.

TABLE 3.—*Analysis of variance for experiments 37, 40, and 46*

Sources of variation	Degrees of freedom	Total squares	Mean square	A/B	Natural logarithm	z
Experiment no. 37: [1]						
Rows	9	6.621				
Strains	51	22.401	0.43924(A)	6.206	1.8255	0.9128
Remainder (error)	459	32.4882	.07078(B)			
Total	519	61.5102				
Experiment no. 40: [2]						
Rows	4	0.1816				
Strains	54	103.0187	1.9077 (A)	5.8300	1.7630	.8815
Remainder (error)	216	70.6759	.3272 (B)			
Total	274	173.8762				
Experiment no. 46: [3]						
Rows	9	1283.41	142.601			
Strains	31	7922.79	255.574(A)	3.693	1.3064	.6532
Remainder (error)	279	19308.50	69.206(B)			
Total	319	28,514.70				

[1] Standard error of means of 10 replicates $=\sqrt{0.07078}\div\sqrt{10}=0.084$ g; difference between means required for significance $=0.084\times2\sqrt{2}=0.24$ g.

[2] Standard error of means of 5 replicates $=\sqrt{0.3272}\div\sqrt{5}=0.256$ g; difference between means required for significance $=0.256\times2\sqrt{2}=0.72$ g.

[3] Standard error of means of 10 replicates $=\sqrt{69.206}\div\sqrt{10}=2.63$ g; difference between means required for significance $=2.63\times2\sqrt{2}=7.44$ g.

In view of the fact that even the parasitically mildest strains in figure 9 caused noticeable rootlet rotting, the surprisingly large and significant differences between these and the severe classes appear to reflect mainly quantitative rather than qualitative differences in virulence. Nevertheless, a comparison of experiments 37 and 40 (on the same variety) suggests that parasitic ability of some of the strains has been differentially modified by unknown qualitative factors of the successive experiments. For example, L–1279 and L–1295 caused reductions that were barely significant in experiment 37 but were among the most virulent in experiment 40. Certain other strains (K–20, L–11, and L–233) were relatively mild and still others (I–(2)132 and L–318) were consistently severe in all tests. There is no segregation according to original host or geographic origin, as shown by the temperature studies, the mild and severe groups here containing both corn and cane representatives. Both large and small oogonial types are found throughout the parasitic range. Despite the wide and significant difference in virulence between isolates at the extremes of the graphs, the number of intermediate representatives and the absence of positive correlation with other physiologic or with morphologic characters such as spore size preclude any practical advantage in subspecific classification of strains.

The fairly consistent behavior of some of the strains on both host varieties is encouraging from the standpoint of testing for resistance, but more extensive data on additional varieties are required to confirm such uniformity as well as to explain the anomolous behavior of other strains in these tests. Johann et al. (*9*) report wide differences in virulence of a single strain toward different inbred lines and open-pollinated varieties of corn. The results of the present study suggest that in order to breed fully resistant varieties the disease-

reaction studies might have to be extended, depending upon the locality, to include several strains of the fungus.

Infection Experiments on Sugarcane

In greenhouse tests all the strains originating in the United States that were listed in table 1 have demonstrated ability to infect sugar-

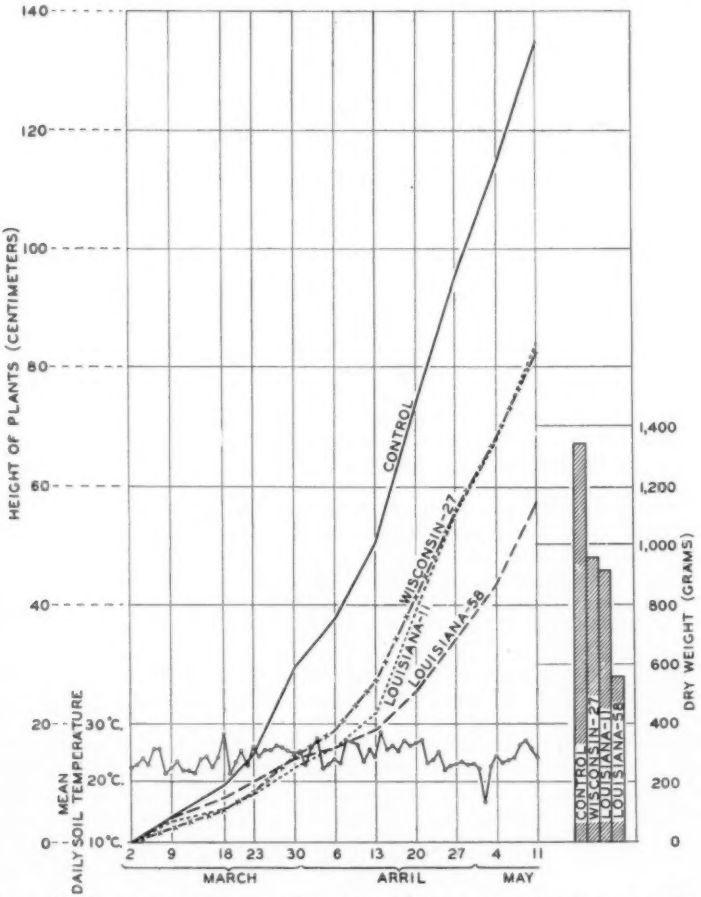

Figure 10.—Mean weekly elongation and dry weight of tops (after 10 weeks) of the Louisiana Purple variety in a soil-compost mixture inoculated with 2 strains of *Pythium arrhenomanes* from sugarcane and 1 from corn (experiment 21).

cane. The remaining isolates, as already mentioned, also proved parasitic in small-scale laboratory experiments. Preliminary qualitative tests, instituted mainly as an aid to identification before oospores had been observed, have invariably revealed large differences in virulence among the strains. On the basis of such preliminary evidence on sugarcane a rough classification into mildly and

severely parasitic groups was made. Cultures L–11 and L–58, among the first isolated in Louisiana, and the type culture W–27 from corn were usually included as controls. L–11 and W–27 had been noted to act similarly in culture, particularly in sporulation on carrot agar, whereas L–58 was always more rapid in mycelial growth and for 5 years persistently failed to produce oospores, until finally tested in humus-extract agar. L–11 is representative of the mildly parasitic isolates and L–58 of the virulent groups.

EXPERIMENT 21 AND EXPERIMENT F

Two quantitative infection experiments in the greenhouse, in which the above-mentioned cane strains were compared with W–27 from corn, are summarized in table 4. Both tests were conducted in Leonardtown silt loam to which about 5 percent of well-composted manure had been added. The mixture was autoclaved at 15 pounds pressure for 2 hours on successive days, remixed, and brought up to and maintained at a moisture capacity of about 70 percent. Other conditions, such as size of container, host employed, temperature, and duration of the tests, were dissimilar. In experiment 21, the very susceptible Louisiana Purple variety was grown in 10-gallon garbage cans under fluctuating greenhouse temperature, whereas experiment F was conducted in constant-soil-temperature tanks of the Wisconsin type, held for an initial period at 16° C. ± 1°, and later raised to 20°, in imitation of winter temperatures in Louisiana. Although in the latter experiment the somewhat more resistant P.O.J. 213 was used, the less favorable soil temperatures permitted about the same percentage reduction for both varieties.

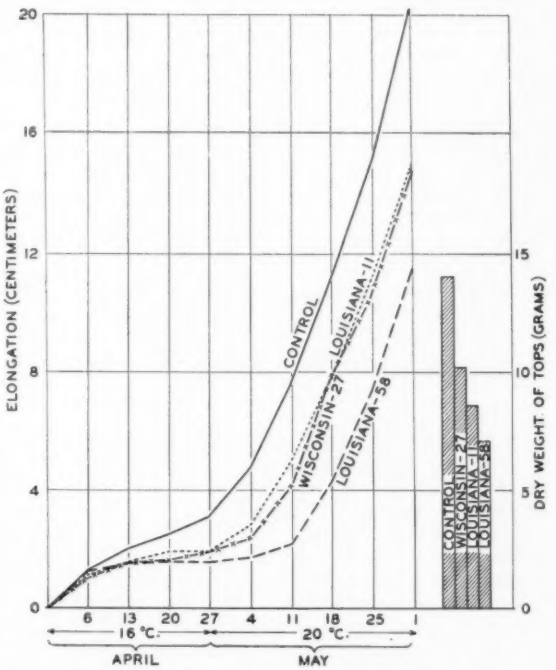

FIGURE 11.—Mean weekly elongation and dry weight of tops (after 2 months) of the P.O.J. 213 variety grown at constant soil temperatures and inoculated with 2 strains of *Pythium arrhenomanes* from sugarcane and 1 from corn (experiment F).

Table 4 and the supplementary data on growth and dry weight given in figures 10 and 11 show that L–58 is significantly more virulent than W–27 toward sugarcane ($P=0.01$). On the very susceptible Louisiana Purple (fig. 10) L–11 and W–27 were not significantly different in effect ($P=0.7$ to 0.8), but on the more resistant P.O.J. 213 (fig. 11), while the plants were practically identical in height, L–11 was intermediate between L–58 and W–27 in weight reduction.

TABLE 4.—*Relative virulence of 2 selected strains of Pythium arrhenomanes isolated from sugarcane and the type culture (W–27) from corn, compared on 2 varieties of sugarcane*

LOUISIANA PURPLE (EXPERIMENT 21)[1]

Strains compared	Plants	Mean height of plants		Dry weight of tops	
		Actual	Reduction due to inoculation	Actual	Reduction due to inoculation
	Number	*Cm*	*Percent*	*Grams*	*Percent*
Control	9	134.7		1,345.8	
L–11	7	84.4	37.3	918.6	31.7
L–58	4	57.4	57.4	562.8	58.2
W–27	6	82.8	38.5	960.0	28.7

P.O.J. 213 (EXPERIMENT F)[2]

Control	16	20.2		14.0	
L–11	16	14.9	26.2	8.5	39.3
L–58	16	11.5	43.1	7.0	50.0
W–27	16	14.6	27.7	10.2	27.1

[1] Mar. 2 to May 3, 1931. [2] Apr. 1 to June 1, 1931.

SAND-CULTURE INFECTION COMPARISONS (EXPERIMENTS 44 AND 45)

In order to secure more reliable quantitative comparisons of strains and more nearly reproducible results, the following sand-culture technic for infection experiments on sugarcane in the greenhouse was employed. Seven- to nine-month-old cane stalks, uniform in size and length of internode, were segmented into single-node cuttings, which were planted in a high-grade autoclaved quartz sand in 4-inch clay pots and watered with a nutrient solution. These were incubated 3 to 5 days at the optimum temperature (35° to 37° C.) for germination and later when the plants were 10 to 14 inches tall, those most uniform in size and vigor were selected and transplanted, without disturbing the roots, to 3-gallon glazed crocks of sterile sand for subsequent inoculation and comparison of fungus strains. At this stage of growth the young plants have initiated a permanent root system (shoot roots) so that should the *Pythium* immediately destroy the temporary or cutting roots (as happens so frequently under field conditions) and molds from air-borne spores ferment the cuttings, the plants are still able to survive. Therefore, in these particular greenhouse tests the study was limited to the effect of the *Pythium* on the permanent root system as manifested by differences in rate of growth and in green or dry weight of tops.

A mineral nutrient solution D containing the following salts in partial volume-molecular concentration was added as required for rapid and normal growth: NH_4NO_3, 0.00225; $Ca(NO_3)_2+4H_2O$,

0.00225; KH_2PO_4, 0.0015; K_2HPO_4, 0.0005; $MgSO_4+7H_2O$, 0.001; KCl, 0.0025 plus rarer elements as earlier listed in this paper for the nutrient solution employed in similar tests on maize. The hydrogen-ion concentration of the fresh solution was pH 6.01, which with weekly renewal and flushing during the course of 2 to 5 months was usually reduced by the plants to between pH 6.2 and 6.6. A 2-quart mason jar set beneath each crock received the leachings by means of a rubber tube from the hole in the bottom of the crock. During the first few weeks of the experiments and between the weekly additions of fresh nutrient, the leachings were poured over the crocks daily, and later twice daily after making up to original volume with distilled water the amount lost by transpiration and evaporation. While somewhat laborious, this procedure maintained a more or less uniform concentration of salts in the sand, besides simulating the effect of rain in possible spread of zoospores and infection throughout the root system.

The object of experiment 44 was to compare the relative virulence toward sugarcane of 8 isolates of *Pythium arrhenomanes* which had shown wide differences in behavior toward corn in experiments 37 and 40. I–(2)132, L–318, and L–1323 had demonstrated a high degree of virulence, whereas L–11, L–58, L–730, F–23, and W–27 (type culture) were only mildly parasitic toward the inbred line of corn used in those experiments. However, it will be recalled that L–58, while relatively mild toward corn, had shown (table 4) greater virulence toward sugarcane than L–11 or W–27. Therefore, it was hoped to check these results as well as to classify the behavior of the remaining cultures toward sugarcane.

TABLE 5.—*Results of sand-culture inoculation test (experiment 44), comparing eight strains of Pythium arrhenomanes on a susceptible and on a resistant variety of sugarcane*

Inoculum used (strain designation)	Mean total elongation per plant of—				Mean green weight per plant of—			
	Louisiana Purple		C.P. 807		Louisiana Purple		C.P. 807	
	Actual	Difference (±controls)	Actual	Difference (±controls)	Actual	Difference (±controls)	Actual	Difference (±controls)
	Cm	*Percent*	*Cm*	*Percent*	*Grams*	*Percent*	*Grams*	*Percent*
None (check)	62.3	-------	213.2	-------	438.1	-------	649.0	-------
L–11	70.2	+12.7	210.4	−1.3	427.1	−2.5	628.9	−3.1
L–58	65.8	+5.6	194.0	−9.0	458.5	+4.7	646.3	−.4
L–318	65.3	+4.8	205.3	−3.7	410.3	−6.3	637.1	−1.8
L–730	64.5	+3.5	213.6	+.2	422.2	−3.6	661.1	+1.9
L–1323	53.7	−13.8	192.7	−9.6	335.4	−23.4	591.5	−8.9
F–23	55.0	−11.7	196.1	−8.0	368.5	−15.9	609.8	−6.0
I–(2)132	64.4	+3.4	203.0	−4.8	409.2	−6.6	607.3	−6.4
W–27	68.0	+9.1	207.6	−2.6	427.1	−2.5	645.1	−.6

As test varieties the very susceptible Louisiana Purple and the very resistant C. P. 807 were employed. One plant of each, germinated as previously described, was transplanted to each crock on May 9, 1933. By June 1, the plants had become well established and were inoculated from 5-day old sand-corn meal cultures of the 8 fungi, 6 crocks

per fungus, with 6 additional crocks of each variety treated with a like quantity of sterile culture medium serving as controls. Inoculated and control crocks were then distributed at random to equalize uneven illumination in different parts of the greenhouse. Aside from a slight reduction in tillering, caused by shading of the house, the plants grew normally, and when harvested August 22, 1933, most of the C.P. 807 plants had attained a height of nearly 5 feet. Mean total elongation and green weights of tops per crock are presented in table 5.

The results in table 5 are in general disappointing for the purpose of strain comparison, because of the relatively slight root damage on even the susceptible variety. Although when the roots were washed out, infection was noted to be general throughout every inoculated culture, rotting, except in the L–1323 and F–23 cultures, was usually confined to the rootlet tips, a typical midsummer condition in commercial fields in Louisiana. Therefore the measurable but unimportant reduction in green weight by most of the strains is believed to have been due to the continuously high temperatures prevailing throughout the test. Temperature readings taken 3 times daily, at a depth of 4 inches from the surface of the sand, gave daily means ranging from 27° to 33° C. and frequent individual readings of 38° to 39°. The usual cool spells during June and July, typical of the climate of Washington, D.C., were in 1933 apparently of insufficient duration to retard the cane plants or to permit the fungi seriously to impair the root system. Thus, imitation of winter and spring soil temperatures in Louisiana was not obtained in this experiment, and the splendid growth of the inoculated plants under optimum temperatures inferentially explains the remarkable recovery and rapid development of initially backward commercial fields following the onset of hot weather.

Table 5 shows that L–1323 and F–23 caused the greatest reduction in green weights. The reductions are statistically significant only for the L–1323 and only on the Louisiana purple variety. ($P = 0.01$, by the pairing method.) Therefore, L–1323 apparently may be considered physiologically different from the others in maintaining relatively greater virulence at high temperatures. L–58, shown in a previous test (table 4) to be extremely virulent on Louisiana Purple at suboptimum temperatures for cane growth, was here relatively harmless.

Repetition of the preceding experiment seemed desirable in order to compare the strains under temperature conditions approximating more closely those prevailing during the critical root-rot period in Louisiana. Thirteen isolates, including the type culture (W–27) from corn, which in preliminary tests had been provisionally classified as either mildly or virulently parasitic toward sugarcane, were compared on two moderately susceptible varieties, Co. 281 and P.O.J. 213. The former was included because during recent years it has become one of the most extensively cultivated varieties, and occasionally its yields on the heavier clay soils have been seriously reduced by root rot.

The experiment (no. 45) was begun October 24, 1933, by setting one plant of each variety in each crock and employing 10 crocks per isolate and 10 controls for each variety. Inoculations were made

October 25, and the various replicates of isolates and controls distributed in randomized checkerboard arrangement on the greenhouse racks. The plants were harvested January 17, 1934, after a growth period of approximately 3 months. At that time the controls and less seriously retarded inoculated series had developed from 3 to 5 suckers each, ranging in height from 3 to 14 cm, while the severely retarded ones were either devoid of suckers or had at most 1 to 2 suckers visible above the surface of the sand. The height of primary shoots and of all suckers was measured weekly and the mean for each series plotted on a graph. Only the successive measurements for the Co. 281 plants are shown in figure 12, along with air temperatures (calculated from daily maxima and minima on thermograph records) and sand temperatures at a depth of 3 inches, read three times daily. Mean total elongation of plants and green weights per series, together with a statistical analysis of the detailed weight data, are presented in table 6. The percentage reductions in green weight of tops (which included also the underground stem, or rhizome) are shown graphically in figure 12.

TABLE 6.—*Comparison of 13 isolates of Pythium arrhenomanes in reduction of yield of 2 moderately susceptible varieties of sugarcane in experiment 45*

[Data represent averages of 10 plants per isolate]

Inoculum used (strain designation)	Total elongation per plant				Green weight per plant			
	Co. 281		P.O.J. 213		Co. 281		P.O.J. 213	
	Actual	Difference (± controls)	Actual	Difference (± controls)	Actual	Difference (± controls)	Actual	Difference (± controls)
	Cm	*Percent*	*Cm*	*Percent*	*Grams*	*Percent*	*Grams*	*Percent*
None (check)	32.8		26.7		181.6		156.5	
L-11	29.6	−9.8	24.4	−8.6	159.5	−12.2	130.3	−16.7
L-58	19.6	−40.2	21.9	−18.0	111.7	−38.5	98.9	−36.8
L-318	22.6	−31.1	20.0	−25.1	127.4	−29.8	103.4	−33.9
L-352	29.4	−10.4	27.7	+3.7	155.0	−14.6	136.4	−12.8
L-382	25.1	−23.5	23.6	−11.6	136.1	−25.1	124.2	−20.6
L-730	30.8	−6.1	26.2	−1.9	155.8	−14.2	135.0	−13.7
L-780	23.9	−27.1	21.2	−20.6	120.8	−33.5	105.8	−32.4
L-803	30.4	−7.3	22.7	−15.0	163.1	−10.2	122.8	−21.5
L-815	23.8	−27.4	20.4	−23.6	124.9	−31.2	103.4	−33.9
L-1141	24.3	−25.9	20.3	−24.0	136.7	−24.7	103.3	−34.0
L-1323	22.1	−32.6	18.6	−30.3	113.2	−37.7	97.1	−38.0
F-23	20.6	−37.2	20.5	−23.2	117.5	−35.3	106.7	−31.8
W-27	30.2	−7.9	26.6	−.4	177.7	−2.1	140.1	−10.5
Standard error of mean					11.008	6.1	11.323	7.2
Difference required for significance					31.130	17.1	32.000	20.4
Mildly parasitic strains (L-11, L-352, L-730, L-803, and W-27)					162.2	10.7	132.9	15.1
Severely parasitic strains (L-58, L-318, L-382, L-780, L-815, L-1141, L-1323, and F-23)					123.5	32.0	105.4	32.7
Difference between mild and severe groups					38.7		27.5	
Standard error of difference [1]					5.377		4.173	

[1] Computed from separate standard errors of group means.

Weekly elongation of inoculated and control plants (fig. 12) was not appreciably different until suckering began (about Nov. 30) and until the sand temperature had dropped to about 20° C. or lower.

Even then the visible manifestation of root damage was not marked by any retarded elongation of primary shoots but rather by the difference in sucker development. Some of the severely damaged plants were also noticeably spindling, which of course was not revealed by

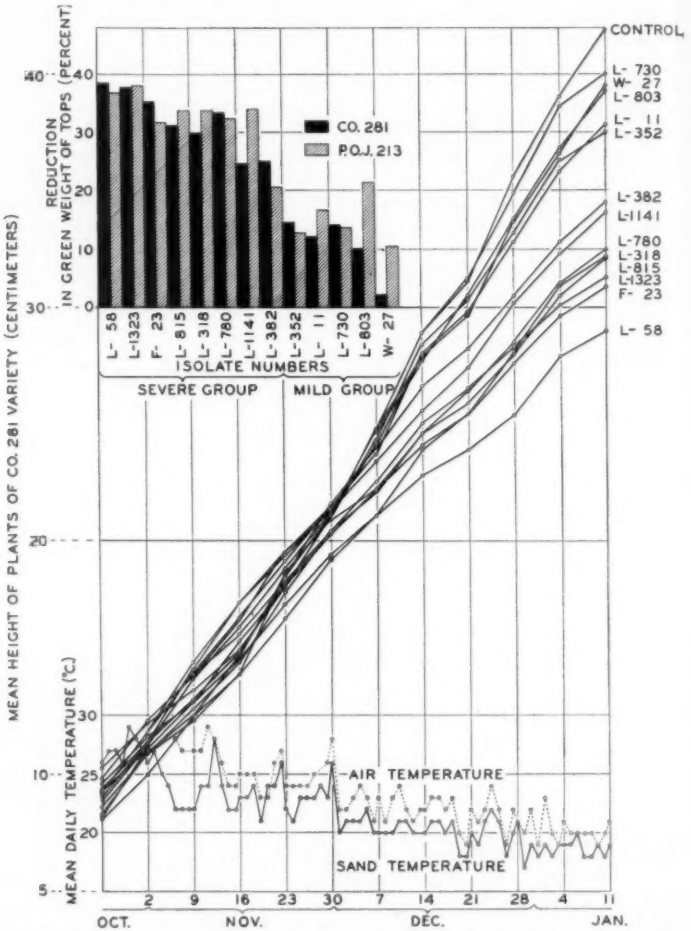

FIGURE 12.—Comparative growth curves of Co. 281 and final percentage reduction in weight of tops of the Co. 281 and P.O.J. 213 varieties from inoculation with 13 isolates of *Pythium arrhenomanes* (experiment 45).

height measurements alone, although in general the growth curves, based on all shoots, foretold fairly accurately the order of plant-weight reduction.

On P.O.J. 213 the reductions in yield due to L–11, L–58, and W-27 are of about the same relative order as those obtained for this variety when planted in soil (experiment F, table 4). Weights of inoculated

series (table 6) and percentage reductions (fig. 12) as compared with controls confirm the preliminary experiments in showing wide differences in virulence between the two groups of isolates selected. However, it may be noted that two members of the "severe group" are revealed as nearly intermediate in virulence toward the varieties employed in this experiment. Because of great variability among the individual replicates of practically all series, the apparent reductions by most of the mild forms are not statistically significant when analyzed by the variance method. However, an examination of roots revealed fairly general infection by the latter, which produced a pinkish coloration of the cortex but which actually rotted only a small proportion of the rootlet tips and caused no apparent reduction in total root mass. The severe forms not only invaded the cortex but tip-rotted the main roots and completely destroyed most of the finer rootlets.

The statistical comparison (table 6) of mild and severe groups reveals in the case of both varieties differences between group means which exceed by approximately seven times the respective standard errors of the differences. This group difference is significant in its magnitude and in the number of isolates making up the respective groups, which, as in the larger experiments on corn and in other physiological tests, suggest an abnormal distribution and the probable existence in nature of specialized subpopulations.

The varietal columns in figure 12 show in general that both varieties were damaged to about the same extent by each isolate. However, certain apparent exceptions may be noted: Inoculations with L–803, L–1141, and W–27 show much greater yield reduction of P.O.J. 213 than of Co. 281. Since one plant of each variety was grown (without crowding) in every crock, the reliability of such apparent differences may be determined by comparing each of these unusual differences with the average difference between the varieties for all 13 fungi. Table 6 shows that Co. 281 showed higher average green weight per plant than P.O.J. 213 in the controls and in all the inoculated series. Thus, the average difference between varieties in the inoculated series is found to be 22.5 in favor of Co. 281. The standard error of this mean is 2.74 g. The deviation of 17.8 g produced by L–803 and of 15.1 g by W–27 from the above-mentioned mean difference exceeds the standard error by 6.5 and 5.5 times, respectively. Therefore, these two isolates at least (and probably also L–1141, with a deviation of four times the error) were significantly more virulent on P.O.J. 213 than on Co. 281. Undoubtedly more exceptions would have appeared had the comparison been between varieties of widely different susceptibility. But it is worthy of note that on these two moderately susceptible varieties the most virulent isolates have acted similarly. Several of these are shown in figure 9 to be also among the most virulent on corn.

DISCUSSION

The oogonial measurements, cultural studies, and infection experiments reported in this paper reveal *Pythium arrhenomanes* to be composed of a considerable number of variously differentiated forms. However, there are enough intermediate or overlapping representatives to invalidate for the present at least any practical advantage of subdividing the species. The extreme variability of the species may

be due to possible hybridization between forms, creating new variants with different morphologic and physiologic characteristics. This is suggested because of the predominantly diclinous relation of antheridia and oogonia (fig. 2). Apposition in plate culture of certain difficultly fruiting isolates usually results in a line of oospores where the different mycelia meet—and sometimes throughout one or both of the thalli of the contacting strains. However, on the types of media that permit fruiting it has not been possible to trace definitely the origin of the antheridia-bearing hyphae.

That all the isolates herein studied belong to this species is obvious from their morphologic similarity alone. The typical crook-necked antheridia and the remote connection between male and female organs (fig. 2) are believed to be more characteristic of the species as a whole than a large number of antheridia, emphasized by Drechsler. Unfortunately, all the relatively few fruiting cultures studied by Drechsler showed large numbers of antheridia, but such are not characteristic of many representatives of the species. A considerable proportion of the isolates included in these studies had typically but 4 to 10 visible antheridia (fig. 2, *D–F*) instead of the 15 to 20 mentioned in the species description. The range in the latter should, therefore, be enlarged to 4 to 20 to include all strains.

As might be expected, the population comparisons reveal no obvious correlation between morphologic groupings and physiologic behavior, and the latter in turn varies with changes in substratum or kind of host. The isolates from the North Central corn-growing States exhibit less tolerance in culture to high temperatures than do the Louisiana and Florida sugarcane strains; they are also less virulent on sugarcane, but some of the sugarcane strains are as virulent on corn as the corn strains themselves. Among the isolates from sugarcane, even from the same field, wide differences in virulence are apparent.

The evidence thus far obtained on physiologic variation indicates that the differences between most of the strains are mainly quantitative rather than qualitative; in other words, differences of degree rather than of kind. However, the inconsistent behavior of a few of the strains that showed wide variation in relative virulence on the same or different varieties of both sugarcane (fig. 12) and corn (fig. 9) suggests the existence of a certain degree of selective pathogenicity. In general the most virulent isolates have acted fairly uniformly on the few varieties tested. Enough cultures belonging to the severe and mild classes have been studied to indicate that such forms are not simply infrequently occurring chance variants of a stable population, but more probably represent genetically different subpopulations in nature. The extent to which one or another might multiply in a given locality would, of course, depend upon the nature of the environment and host. With respect to maize, Johann, Holbert, and Dickson (*9*) have demonstrated differences in varietal resistance to a single strain of the fungus, and the evidence in this paper suggests the importance of taking into account differences among strains on the same variety. It is well known that *Pythium* root rot of corn is important in some States or districts and apparently unimportant in others. The possibility is suggested that this may be due, partly at least, to unequal distribution of physiologic forms of the fungus as well as to differences in varietal resistance or to other factors.

Sugarcane cultivation presents an ideal combination of circumstances for the expression of differences in both varietal susceptibility and parasitic development. Here, for a period of years, through successive plant and ratoon crops, thousands of acres may be occupied by a single variety (vegetative clone) the individual plants of which are genetically identical. Not only might nutritional idiosyncrasies become manifest but profound changes in the biology of the soil eventually occur. Among the latter might be the gradual multiplication of physiologic forms of *Pythium arrhenomanes* and other disease organisms which can successfully attack a newly introduced variety, or the spread of new forms on a long-cultivated variety.

Almost every sugarcane-producing country has witnessed a continual change in varieties, and before the general recognition of diseases, so-called degeneration was a common explanation. While most of the historic failures of cane varieties have been satisfactorily explained by epidemic occurrence of some disease (often an ill-defined root disease) or insect plague, the opinion still apparently persists among growers that a newly introduced variety gradually falls off in yield after prolonged cultivation. Unfortunately, this is a question difficult of experimental analysis, but the continued and increased yields of H–109 in Hawaii represent at least one notable exception. On the other hand, the comparatively sudden failure of Lahaina, which preceded H–109 in that country, was due apparently in part to a *Pythium* root rot of which one isolate included in this study is definitely *Pythium arrhenomanes*. To what extent this particular species contributed to the failure of Purple and D–74 in Louisiana is uncertain, although it is well known that root diseases played an important role. Louisiana has recently witnessed the almost complete failure of P.O.J. 213, which from 1923 to 1928 was considered the most valuable variety introduced up to that time by the United States Department of Agriculture. Abbott (*1*) has demonstrated the existence of physiologic forms of the red-rot fungus (*Colletotrichum falcatum* Went.), the dominant form of which now causes severe injury to P.O.J. 213 and is probably the major cause of its failure. However, there has been a noticeable increase in the apparent damage from root rot, but whether this has been due to possible weakening of the roots from red rot attacking the seed cane and stubbles or to the multiplication of more virulent strains of *P. arrhenomanes* has not been definitely determined.

The isolations of *P. arrhenomanes* made in 1931 by E. M. Summers from the varieties P.O.J. 213, Co. 281, and C.P. 807 are predominantly of the L–58 and L–1323 virulent types (tables 4 to 6), whereas those obtained prior to 1928, mainly from the formerly grown D–74 and Louisiana Purple, are predominantly less virulent on the latter very susceptible varieties. The theory that the behavior of the latter might be due to loss of virulence in long-continued artificial culture is refuted by the consistent behavior of certain representatives in repeated inoculation tests; L–39, L–40, and L–58, for example, were among the first cultures obtained in Louisiana and have remained among the most virulent. Therefore, while the role of *P. arrhenomanes* as an important causative factor in the failure of P.O.J. 213 is problematical, the studies, which are being continued, indicate that the widespread adoption by the Louisiana industry of more vigorous varieties is apparently bringing about a qualitative change in the population of

this species. Should this be confirmed by intensive varietal and regional surveys, the number of certain physiologic forms would be expected to increase rapidly and in time reduce crop production of any newly introduced varieties that are not fully resistant. As an advance safeguard in the breeding program of the United States Department of Agriculture, all promising new seedling canes are being tested for their resistance to several selected strains of the fungus. The industry should always have available several apparently resistant varieties rather than rely on only one or two temporarily outstanding canes. Then, at each replanting, similarly adapted varieties could be successively shifted or rotated among the different fields of the plantation, and thus counteract possible varietal specialization of the root-rot fungi.

SUMMARY

This paper presents the results of laboratory and greenhouse studies on strain variability of the principal sugarcane root-rot fungus, *Pythium arrhenomanes* Drechsler, in Louisiana and Florida. Additional cultures from corn root rot in the North Central States, the cereal root-browning fungus from Canada, and sugarcane strains from Hawaii and Mauritius were included in the laboratory comparisons.

Despite wide differences in size of oogonia, number of antheridia, rate of growth in artificial culture, and response to temperature, and despite variation in hydrogen-ion concentration and in virulence among the local forms on sugarcane and corn, certain constant and distinctive characteristics of the sexual organs unquestionably classify all the 70 isolates tested as *Pythium arrhenomanes*. Nine of the recently described "*Nematosporangium*" species of Sideris (*13*), mainly from pineapple in Hawaii, are also included under the species.

A tentative explanation for the diversity of forms encountered in this species involves the possibility of hybridization between types, which would be particularly favored by the diclinous relationship of antheridia and oogonia.

Physiologic specialization is indicated by the greater tolerance to high temperatures of the sugarcane isolates as compared with isolates from the corn-producing States, and particularly by the great differences in virulence among representatives of each group to both crops.

Although the differences in virulence are of a magnitude commonly employed for differentiating physiologic forms in other fungi, the limited population studies reveal enough intermediate or overlapping cultures to nullify, for the present at least, any practical advantage of subspecific groupings.

Preliminary surveys and infection studies (in progress) indicate an increase in population of the more virulent strains, at least toward the Louisiana Purple variety, since the replacement of the latter and D–74 by more productive types.

Differences in virulence and geographic distribution of strains should be considered in the breeding of resistant varieties. A number of apparently resistant varieties should always be available and as far as practicable successively rotated on the different fields of the plantation to retard possible specialization and multiplication of destructive physiologic forms of *Pythium arrhenomanes*.

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CHROMOSOME NUMBER AND MEIOTIC BEHAVIOR IN GOSSYPIUM [1]

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INTRODUCTION

The literature dealing with heritable variations in cotton (*Gossypium*) was summarized by Kearney (*18*) [2] in a recent paper on the genetics of cotton. Variations due to new combinations are common. This may be attributed to the fact that all cultivated varieties of cotton are genetically impure, comprising numerous heterozygous individuals. It is not unlikely that all the existing cultivated forms are of hybrid origin. The literature dealing with mutational variants in cotton is very meager, and in most of the cases reported the evidence is unconvincing. One of the best authenticated examples is that of white-flowered sea-island described by Harland (*15*). However, the occurrence of rogue plants in inbred lines and the fact that some of these variants exhibit alternative inheritance (*19*) probably indicate that mutations are not infrequent. Where genetic analysis has been possible, all such mutational variants in cotton have proved to be due to alteration in a single gene. As far as the writer is aware, no chromosomal aberrancies have been reported in cotton. Since few cytological observations have been made on this genus and since the majority of chromosomal aberrants have been discovered by cytological means, it is not surprising that such aberrancies have not been reported in the genus *Gossypium*.

Cytological studies on the meiosis of cotton have heretofore been chiefly concerned with the processes of the divisions and the chromosome numbers characteristic of the different species and forms. The behavior of meiotic chromosomes has not been thoroughly studied. Hence, the exact chromosome composition of the gametes is unknown. Since heritable qualities depend upon the chromosome composition of the gametes, it is important that a complete analysis of meiosis should be made. The present paper attempts to determine the chromosomal composition of the gametes produced by the multitudinous forms of cotton. No attempt will be made here to discuss the general cytological literature dealing with cotton. For discussions of chromosome numbers and the processes of meiosis the reader is referred to the discussions and bibliographies of Longley (*20*) and Beal (*3*).

[1] Received for publication Dec. 1, 1933; issued August, 1934.
[2] Reference is made by number (italic) to Literature Cited, p. 236.

MATERIAL AND METHODS

This study has been confined to typical species and forms in the collection of living plants of Hibisceae, at the Rubidoux Laboratory, Riverside, Calif., disregarding such variant individual plants as occurred there. The fixations of buds for study were made between May and October 1932. Three of the first plants examined, 1 exhibiting fairly normal meiotic behavior and 2 exhibiting irregularities, were used as cytological checks throughout the period during which material was collected. Since these three plants continued throughout the season to exhibit the same cytological behavior, the data herein reported for other plants may be assumed to be characteristic of the plants and not due to environmental conditions.

The study was limited chiefly to pollen mother cells prepared from anthers fixed in Carnoy's fluid for 10 to 30 minutes, washed and preserved in alcohol, and crushed in aceto-carmine. Permanent preparations were obtained from anthers fixed in chromo-acetic-formalin and stained in Heidenhain's iron-alum-haematoxylin.

All drawings were made at bench level with a camera lucida having a 1.5-mm objective (1.3 N.A.), an 18× compensating ocular, and a tube length of 160 mm, to give a magnification of 4,650 diameters. They were then reduced one-half in reproduction, so that the magnification as shown is $\frac{4,650}{2}$.

CHROMOSOME NUMBERS OF GOSSYPIUM

The chromosomes of *Gossypium* have been briefly described and figured by several investigators (*3*, *10*, *11*, *20*). Both the meitotic and meiotic chromosomes are unusually small. This condition is particularly noticeable in relation to the large amount of cytoplasm. Although several investigators have described the occurrence of a few large chromosomes within the complement, the writer is inclined to believe that these bodies are nucleolar remnants. The chromosomes are frequently well separated, so that their number is easily counted. There are very few discrepancies in the reported numbers.

Almost simultaneously, Denham (*10*, *11*), Nikolajeva (Zaitzev) (*27*), and Longley (*20*), showed that the Asiatic cottons have 13 pairs of chromosomes and that the cultivated American cottons have 26 pairs. For the chromosome numbers previously reported in *Gossypium*, the reader is referred to Gaiser (*12*, *13*), Tischler (*24*), and Longley (*20*).

The haploid chromosome numbers determined by the writer are given in table 1, column 1.

In table 1, the numbers in column 3 are those under which the seeds were cataloged as received by the Bureau of Plant Industry, with the exception of a few plants grown by the writer from seeds that had not received a "C.B." (cotton breeding) number. These are indicated by the letter W preceding the number. Different numbers under a given species, therefore, represent different introductions and in some cases different forms of the species in question. For example, in the case of *Gossypium nanking* Meyen, plants were examined representing three separate introductions (783, 754, and 393). In most cases, the species names are those under which the seeds were received, and in several cases their correctness is doubtful. The taxonomy of the genus *Gossypium* is in such confusion that identification of many of the forms is very difficult.

TABLE 1.—*Meiotic behavior of various species and forms of Gossypium*

Group and number of chromosomes	Species or form	Catalog no.	Chiasmata		First metaphase plates		Maximum quadrivalents	Second metaphase plates		Maximum chromosomes eliminated	Sporads exhibiting microcytes
			Terminal or subterminal	Interstitial	Total	Exhibiting quadrivalents		Total	Having less than n[1] chromosomes		
			Number	*Number*	*Number*	*Percent*	*Number*	*Number*	*Percent*	*Number*	*Percent*
(1) Cultivated Asiatic cottons, $n=13$.	*G. nanking* Meyen	783	10	2	21	0	0	[2] 22	4.55	1	0
	do	754	10		13	0	0	23	8.69	1	.66
	do	393	9	3	30	0	0	29	10.34	1	2.08
	G. arboreum L.	620	9	2	23	0	0	70	7.14	2	2.28
	G. sanguineum Hassk.	787			13			[3] 13	7.70	1	5.39
	G. neglectum Tod.	786	10	1	17	0	0	31	6.45	2	2.68
	do	784	11	1	16	0	0	21	0		.60
	G. herbaceum L.	743	9	2	40	0	0	19	10.57	2	3.10
	G. cernuum Tod.	422	9	3	22	0	0	83	10.84	3	5.50
	G. intermedium Tod.	619	10		13	0	0	8	0		4.11
	G. obtusifolium Roxb. (India)	765			21	0	0	23	8.69	1	4.15
	G. obtusifolium Roxb. (Algeria)	749	8	2	30	0	0	[3] 36	8.33	1	3.85
(2) Wild Australian species, $n=13$.	*G. sturtii* F. Muell.	632			2	0	0	9	0		
	do	564	12 or 13		14	0	0	15	0		0
	G. klotzschianum Anders.	700						8	0		0
(3) Wild American species, $n=13$.	*G. harknessii* Brandeg.	861						5	0		1.31
	G. armourianum Kearney	867						13	0		
	G. davidsonii Kellogg	101	13?		39	0	0	39	0		0
	G. peruvianum Cav.	662	19	3	48	10.42	2	22	4.50	1	1.96
	G. barbadense L.	W5			3			21	0		0
	G. brasiliense Macf.	673	20	4	33	12.10	2	27	3.70	1	0
	G. darwinii Watt	636			3	0		6	0		0
(4) Cultivated and related wild cottons, South American type, $n=26$.	do	709			9	11.11	1	14	0		.66
	G. tomentosum Nutt.	527			3			7	0		1.32
	G. sp.	204a			2			2	0		.05
	do	204b			4			23	4.35	1	.99
	do	231b			3			10	0		0
	G. hirsutum L.	723	18	5	53	9.43	5	22	9.09	1	2.91
	do	W12			5			4	0		.49
	do	W6	20	4	4			[4] 34	2.93	2	1.06
	G. contortum Cook and Hubbard	542			6			4	0		0
	G. patens Cook and Hubbard	541			5	20.00	1	40	10.00	3	2.23
	G. morrilli Cook and Hubbard	396			8	12.50	1	4	0		0
(5) Cultivated and related wild cottons, North American type, $n=26$.	*G. punctatum* Schum. and Thon	437	21	3	6	16.66?	1?	20	5.00	1	1.00
	G. jamaicense Macf.	670			15	6.66	2	32	0		.66
	G. hopi Lewton	716	19	4	23	8.70	2	22	0		.49
	G. schottii Watt	672	21		27	3.70	1	15	0		0
	G. sp. (Mexico)	750			1						2.44
	G. sp. (Florida)	421			10	10.00	3	17	5.90	1	4.95
	do	644			3			31	3.23	1	1.96
	do	W16			23	8.70	2	32	9.38	2	3.85

[1] Haploid chromosome number.
[2] 1 second metaphase plate had 13 chromosomes plus 1 fragment.
[3] 1 pollen mother cell not recorded in table had second metaphase plates containing 12 and 14 chromosomes.
[4] 1 pollen mother cell not recorded in table had second metaphase plates containing 28 and 24 chromosomes.

The haploid chromosome number 13 for all the species in the Asiatic group is in agreement with that previously reported for these species.

Although the four wild American species listed in table 1 have 13 pairs of chromosomes, as the Asiatic cottons have, they are very distinct phenotypically from the Asiatic cottons and from each other. Longley (*20*) reports *G. armourianum*, no. 862,[3] from San Marcos Island, Baja California, as having 13 pairs of chromosomes. Harland (*14, v. 6*) and Longley (*20*) report 13 as the haploid chromosome number of *G. davidsonii*. In a letter to Dr. T. H. Kearney, dated May 15, 1931, Dr. S. C. Harland wrote: "No. 700 is close to *G. klotzschianum*. It has 13 chromosomes and is rather like a giant *davidsonii* in appearance."

Gossypium sturtii, a native of Australia, is reported by Harland (*14, v. 6*) to have 26 pairs of chromosomes, and by Longley (*20*) to have 13 pairs. The writer's determination agrees with that of Longley.

The haploid chromosome number 26 for all species and forms of the South American group is in agreement with that found by other investigators. In this group the writer has included *Gossypium darwinii* and *G. tomentosum*. In regard to the writer's determination of the chromosome number in *G. darwinii*, Dr. Kearney commented as follows:

> It is interesting to know that, while *G. klotzschianum* has 13 chromosomes, *G. darwinii* has 26. This is in line with my observations of the morphological characters of the two species, *G. darwinii* evidently being much more nearly related to the cultivated cottons of the South American group than is *G. klotzschianum*.

So far as the writer knows, no report of chromosome number in *Gossypium darwinii* has been published hitherto. Dr. Harland places *G. tomentosum* with the American cottons and states that it probably has 26 pairs (*14, v. 5*). Regarding this species, in a letter dated December 29, 1932, Dr. Kearney wrote:

> Whether it belongs to the South American or the Mexican series I am unable to say, although I suspect it is more closely related to the former group. I have never seen it in flower or fruit.

The haploid chromosome number 26 for all species and forms of the North American group is also in agreement with that reported by previous authors. In this group are included several wild cottons from Florida. Since these wild plants exhibit cytological behavior similar to that of cultivated forms of this group, and since their history is of scientific value, the following notes [4] regarding the parent plant of one of them (W16) are given here:

> In 1907 or 1908 [H. S. Fawcett] saw a large cotton plant with a trunk 5 inches in diameter growing on an island off from Mr. B. L. McMullen's Badwater place about 6 or 7 miles south of Bayview, Fla. This plant with large trailing branches supported by small trees and bushes then had an estimated spread of 50 feet. In 1932 [Fawcett,] with Dr. H. J. Webber again visited the island and found the old trunk partly decayed but with a shoot coming up from the base. There were also a number of separate cotton plants with long branches, growing up into the bushes. The space occupied was about 65 or 70 feet in diameter and had few other plants growing in the center of the area but was surrounded by

[3] Reported by Longley under the name *G. harknessii*, but this form has since been recognized as a distinct species. The writer has determined the occurrence of 13 pairs of chromosomes in both *G. armourianum* and the true *G. harknessii*.

[4] Communicated by Dr. H. S. Fawcett, Citrus Experiment Station, Riverside, Calif.

a thick jungle of bushes and vines. Several cottons were apparently growing well, quite near where salt water came up at high tide.

Dr. Fawcett states in a letter dated January 12, 1933: "I think one could safely estimate that this plant had been in its present location for at least 50 years, if not more."

Gossypium kirkii Mast., which is not reported in table 1, probably has 12 pairs of chromosomes. According to Watt (*25*), this species inhabits east and central Africa and has never been seen there under cultivation. In a communication of October 1930 the writer reported to Dr. Kearney that this species has either 24 or 26 somatic chromosomes. In an annotated copy of a recent publication Harland (*16*) states that Skovsted found the haploid number of chromosomes in *G. kirkii* to be 12.

MEIOSIS IN GOSSYPIUM

The statements in this paper regarding meiotic phenomena are limited to interpretations within groups of species rather than of individuals or forms. Since a study of the thread stages of *Gossypium* has not yet been completed, only the behavior from diakinesis onward will be discussed.

During early diakinesis the chromosomes are usually well separated and exhibit slight variations in size and shape. Although the chiasmata are apparent, no study of them has been made at this stage. The chromosomes of the Asiatic species, the wild American species, and *Gossypium sturtii* are always bivalent. Hence, the characteristic haploid number, 13, is readily determinable. On the other hand, in the cultivated American species, the chromosome counts are not always 26 but range from 22 to 26, with 26 preponderating. Nuclei having fewer than 26 chromosomes exhibit quadrivalents, but not more than 4 quadrivalents have been observed in such nuclei.

Later in diakinesis the chromosomes are considerably smaller and show less variation in shape. The latter condition is probably due to a slight terminalization of interstitial chiasmata, produced by contraction. The chromosomes at this stage also tend to mass, especially in the species having 26 pairs.

During the first metaphase (figs. 1–7) a few of the chromosome pairs are usually obscured by others or are situated at an angle which makes them difficult to study. Hence, it is rather toilsome to obtain a complete picture of the chiasmata (*8*) of any single species. In the following paragraphs, the given ranges of chiasma types occurring within a group of species are the maximum ranges observed but may not represent the absolute maxima. However, the observed ranges indicate the relative proportions of chiasma types occurring within a group and between the groups.

In the Asiatic cottons (figs. 2 and 3, *A-U*) apparently from 8 to 11 (table 1, column 4) of the paired chromosomes are attached by terminal or subterminal chiasmata. Only one of the wild American species, *Gossypium davidsonii* (fig. 4), has been studied from this point of view. All the bivalents of this species and of *G. sturtii* are attached by terminal or subterminal chiasmata. From 18 to 21 of the paired chromosomes of the cultivated American species are attached by these types of chiasmata (fig. 1, *A-U*). Chromosomes attached by subterminal chiasmata (figs. 1–3, *E*, *F*, *M-U*) are easily distinguishable by small free ends or knoblike protuberances from those attached by terminal chiasmata (figs. 1–3, *A-D*, *G-L*).

In *Gossypium*, interstitial chiasmata are usually close to the ends of the bivalent so that they are difficult to separate from subterminal chiasmata. However, in nearly all species a few pairs have the attachments removed from the chromosome ends (figs. 1–3, *V–Z*). Figure 1, *Z*, depicts a configuration which is attached by all three types of chiasmata and is frequently observed in the majority of cottons. From 1 to 3 of the bivalents of the Asiatic cottons [5] and from 3 to 5 of the bivalents of the cultivated American cottons exhibit interstitial chiasmata (table 1, column 5). As indicated above, no interstitial chiasmata have been observed in either *G. davidsonii* or *G. sturtii*.

FIGURE 1.—*Gossypium hirsutum* (723). Lateral view of first metaphase in slightly crushed pollen mother cell, showing chiasma types: *A–C, G–L*, Terminal chiasmata; *E, F, M–T*, subterminal chiasmata; *V, W*, interstitial chiasmata; *Z*, all three chiasma types.

FIGURE 2.—*Gossypium cernuum* (422). Lateral view of first metaphase in slightly crushed pollen mother cell, showing chiasma types: *A, G–J*, Terminal chiasmata; *E, M, N, S*, subterminal chiasmata; *W, Y*, interstitial chiasmata; *Z*, all three chiasma types.

FIGURE 3.—*Gossypium neglectum* (784). Profile view of first metaphase in slightly crushed pollen mother cell, showing chiasma types: *A, G–L*, Terminal chiasmata; *E, M, N, S*, subterminal chiasmata; *X*, interstitial chiasmata.

FIGURE 4.—*Gossypium davidsonii* (101). Lateral view of first metaphase in slightly crushed pollen mother cell, showing chiasma types: *A, B, G–L*, Terminal chiasmata; *E, M–P*, subterminal chiasmata.

Although no attempt has yet been made to determine the exact number of chiasmata in the various species, the number apparently ranges from 1 to 3 per chromosome, with 2 preponderating. The total number per chromosome complement is undoubtedly relatively

FIGURE 5.—*Gossypium darwinii* (709). Polar view of first metaphase in pollen mother cell; 26 bivalents.

FIGURE 6.—*Gossypium hirsutum* (723). Polar view of first metaphase in pollen mother cell; probably 10 bivalents, 6 secondarily paired bivalents, and 5 quadrivalents.

FIGURE 7.—*Gossypium schottii* (672). Polar view of first metaphase in pollen mother cell; 26 bivalents, of which at least 18 are secondarily paired.

[5] The occurrence of 3 interstitial chiasmata in *Gossypium cernuum* (422) and 11 terminal or subterminal chiasmata in *G. neglectum* (784) indicates that the type of chiasma is variable within the Asiatic group.

high for the length of the chromosomes. Darlington (*8*) has shown that because of terminalization the number of chiasmata diminishes between the diplotene and metaphase stages. Whether such a condition exists in cotton cannot be said, since the study of the thread stages is not yet complete. However, the fact that the metaphase chromosomes exhibit a relatively high number of chiasmata for their length suggests that there is no appreciable reduction in the number between the thread stage and early metaphase. Since the majority of bivalents are attached at both ends or near both ends, the majority of configurations are more or less ring-shaped (figs. 1–4, *G–U*). One terminal or subterminal chiasma results in a more or less rod-shaped bivalent (figs. 1–4, *A–F*). Configurations in the form of double rings possess 3 chiasmata, of which 1 is interstitial (fig. 1, *Z*). Double-cross-shaped configurations (figs. 1 and 2, *Y*) possess two interstitial chiasmata.

As the metaphase develops the spindle-fiber constrictions become more apparent and are generally seen to be located at the maximum distance from the chiasmata. The exceptions to the latter rule are to be found in the pairs attached by interstitial chiasmata (figs. 1–3, *V–Z*). In figures 1 and 2, *Y–Z*, the interstitial chiasmata are exceptionally close to the points of spindle-fiber attachment.

FIGURE 8.—Lateral views of first metaphase; quadrivalent configurations: *A'*, *Gossypium* sp., from Florida (W16); *B'*, *C'*, *G. hopi* (716); *D'*, *G. hirsutum* (W12).

Table 1, column 6, gives the number of first-metaphase plates studied for the several species listed in column 2. Column 7 shows the percentage of these plates that contain quadrivalents, and column 8 lists the maximum number of quadrivalents observed in the several plates.

The data in table 1 indicate that the species with 26 pairs of chromosomes occasionally form as many as 5 quadrivalents (figs. 6 and 8), whereas the species with 13 pairs do not form quadrivalents. In the cultivated American groups, a total of 341 plates were studied, of which 26, or 7.62 percent, exhibited quadrivalents. Since this percentage is the average for the group, it probably more nearly approaches the percentage of plates per individual exhibiting quadrivalents than is indicated in table 1.

Quadrivalents apparently exhibit only terminal or subterminal chiasmata. The most common configuration (fig. 8, *A'*) exhibits 2 triple and 1 simple terminal chiasmata. The slight variation in this configuration shown in figure 8, *B'*, is undoubtedly due to the degree of terminalization. Occasionally a configuration occurs that has 1 triple and 2 double chiasmata (fig. 8, *C'*). Since this configuration only occasionally occurs and in plates having other types of quadrivalents, undoubtedly it is a different quadrivalent and not simply a modification of the more usual quadrivalent due to terminalization. The third distinguishable quadrivalent (fig. 8, *D'*) is a ring of four chromosomes, having four simple chiasmata. The latter quadrivalent has been observed only in forms of *Gossypium hirsutum* and then only rarely.

Dr. Darlington has recently called the writer's attention to the phenomenon of secondarily paired bivalents, which undoubtedly

occurs in *Gossypium*. Since this important condition was overlooked during the present study, very few data are available concerning it. However, several drawings clearly support its occurrence in the cultivated American cottons (figs. 6 and 7). In considering the data obtained from such drawings, it seems probable that secondarily paired bivalents occur more frequently than quadrivalents. Such association is apparently always in twos, and may occur in plates that do or do not exhibit quadrivalents. Figure 7 depicts 18 secondarily paired bivalents, and figure 6 contains 6 secondarily paired bivalents in addition to the maximum number of quadrivalents.

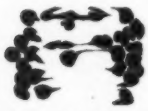

FIGURE 9.—*Gossypium herbaceum* (743). Profile view of abnormal first anaphase in pollen mother cell; 2 lagging bivalents.

The significance of the phenomenon and of the evidence submitted in the preceding paragraphs will be discussed in the concluding section. It might be noted here that the chiasmata of all cultivated species of *Gossypium* are similar in nature and extent.

During the first anaphase the majority of bivalent partners disjoin normally and move toward opposite poles as single units (figs. 9 and 10). It is rather difficult to trace the behavior of quadrivalents. However, they separate regularly into daughter quarters, and in some cases, at least, adjacent partners are distributed to opposite poles (fig. 8). Immediately following disjunction a few chromosomes are occasionally found in the equatorial zone in the cultivated forms of both the American and the Asiatic species (figs. 9 and 10). These chromosomes are bivalent in nature and exhibit a varying degree of separation. Such lagging bivalents usually exhibit 1 or rarely 2 terminal chiasmata. Occasionally, however, they are attached by subterminal or interstitial chiasmata (fig. 9). Later in the anaphase all lagging bivalent partners disjoin and are distributed normally toward the poles, but on account of their delayed separation and movement they are frequently left in the plasma. As many as 5 lagging bivalents in the Asiatic forms and 8 in the cultivated American forms have been observed. Approximately 13 percent of the pollen mother cells of the cultivated American forms and 11 percent of the Asiatic forms exhibit lagging bivalents during the first anaphase.

FIGURE 10.—*Gossypium peruvianum* (662). Profile view of abnormal first anaphase in pollen mother cell; 3 lagging bivalents.

The second metaphase plates are well separated and contain sharply distinct chromosomes. The conditions existing at this stage are summarized in columns 9 to 11 of table 1.

As indicated in table 1, the cultivated forms of both the Asiatic and the American species frequently exhibit second-metaphase plates having less than the haploid number of chromosomes. In the Asiatic group a total of 570 plates were studied, of which 54, or 9.47 percent, contained from 1 to 3 chromosomes less than 13. In the two groups of American cultivated cottons 498 plates were studied, of which 21, or 4.21 percent, contained from 1 to 3 chromosomes less than the haploid number. Very rarely, in all cultivated cottons, second-metaphase plates exhibit 1 or 2 chromosomes more than the characteristic haploid number. (See table 1, footnotes 2–4.) The latter plates

probably resulted from first anaphases in which the homologues of lagging bivalents failed to disjoin and the bivalent was finally distributed to one pole. Figures 11 and 12 depict such irregular or abnormal second metaphases, which may be interpreted as results of first-anaphase distribution and elimination of lagging bivalents.

Although very little study has been made of the second anaphase, several noteworthy conditions have been observed. Occasionally in the plasma a few whole chromosomes are found which exhibit normal division and distribution on miniature achromatic figures. Within the major achromatic figures all chromosomes likewise exhibit normal division and distribution. Although the achromatic figures do not overlap, occasionally one pole of a miniature spindle is adjacent to one pole of a major spindle, so that chromosomes distributed to these poles lie in close proximity. In no case, however, are anaphase groups of chromosomes close enough together to form a single granddaughter nucleus. Such second-anaphase groups from the major and miniature achromatic figures undoubtedly account for the abnormal sporads described later. The following few counts from normal and abnormal second-anaphase pollen mother cells are in accordance with the conditions observed in the first anaphase and second metaphase.

FIGURE 11.—*Gossypium obtusifolium* (749). Polar view of abnormal second metaphase in pollen mother cell; results of a 12<>14 first-anaphase distribution. (Daughter plates shown closer together than they actually occur in mother cell.)

FIGURE 12.—*Gossypium* sp. from Florida (W16). Polar view of abnormal second metaphase in pollen mother cell; results of a 24<4>24 first-anaphase distribution. (Daughter plates shown closer together than they actually occur in mother cell.)

4 counts of $13<>13+13<>13$; 2 counts of $13<>13+\overset{1}{\underset{1}{\frown}}+12<>12$;

2 counts of $12<>12+\overset{2}{\underset{2}{\frown}}+12<>12$;

2 counts of $26<>26+26<>26$;

and 1 count of $25<>25+\overset{2}{\underset{2}{\frown}}+25<>25$.

The twelfth column in table 1 gives the percentage of sporads containing more than the normal number of four spores. In no case are sporads formed having less than four spores. The spores of the tetrads are equal in size. Sporads containing more than 4 spores generally exhibit 1 to 3 additional small spores or microcytes. However, not infrequently sporads contain 8 or 9 spores, which are equal or nearly equal in size to each other and to the spores of the tetrads.

Although no evidence has been obtained as to the mode of formation of the latter highly abnormal sporads, they may possibly arise from binucleate pollen mother cells (*26*). Tetrads, as well as the other types of sporads, occasionally exhibit micronuclei.

In each case the percentage of abnormal sporads recorded in table 1 is based on 400 or more counts. Hence, these percentages represent fairly accurately the relative frequency of irregularities exhibited by the various species and forms. As indicated in the table, the sporads of the cultivated species generally exhibit irregularities, while those of the wild species do not. The sporads of the Asiatic species are more highly abnormal than those of the cultivated Ameri-

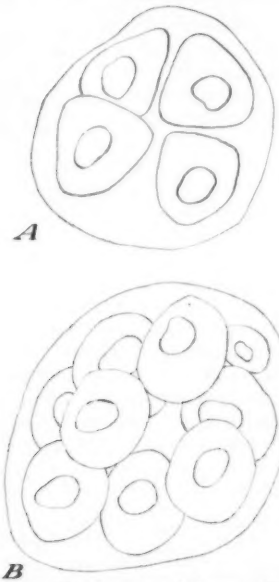

FIGURE 13.—*Gossypium herbaceum* (743). Sporad types: *A*, Normal tetrad; *B*, abnormal sporad. Note that the spores of *B* are nearly equivalent in size to those of *A*. × 245.

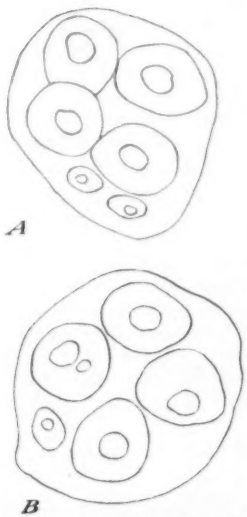

FIGURE 14.—Sporad types, showing microcytes and micronuclei: *A*, *Gossypium cernuum* (422); *B*, *G. hirsutum* (723). × 245.

can forms. The table also indicates that the percentage of abnormal sporads varies within the group and within the species.

DISCUSSION

CHROMOSOME NUMBER AND MORPHOLOGY

As stated in the previous section, there are very few discrepancies in the reported chromosome numbers of cotton. Discrepancies that have been reported probably are due to misinterpretations of stages and of irregularities. In several instances, in both meitotic and meiotic metaphases, the writer has observed nucleolar remnants which might easily be mistaken for chromosomes. Denham (*10*) reports the occasional occurrence of diploid diakinesis and of apparently diploid

first anaphases. Such irregularities and the additional occurrence of quadrivalents in the cultivated American forms, make it difficult to ascertain the characteristic chromosome numbers without numerous counts.

Opinions differ as to the heterogeneity in size of the chromosomes within a given species of *Gossypium*. In *G. barbadense*, Denham (*10*) finds two chromosomes which are distinctly larger than the others. In the same species, Beal (*3*) finds no considerable variation in size, but a "uniform gradation in size from very small to relatively large chromosomes, * * * with 5 or 6 larger than the others." Banerji (*1*) finds no appreciable differences in the size of the chromosomes of Indian cottons. Since the majority of Denham's figures show no appreciable size differences and since his figure 1 (*11*), which depicts the greatest differences in size, contains more than 26 chromosomes, the writer believes he mistook nucleolar remnants for large chromosomes. Whether appreciable differences in size of the chromosomes exist in cotton is questionable. Camera-lucida drawings of polar views of meiotic metaphases, such as figured by Beal (*3*), certainly show a gradation in chromosome sizes. Since, however, study of such plates shows clearly that the chromosomes are at different levels within the plate, these drawings are misleading. Furthermore, in all such polar views a detailed study shows that the bivalents exhibit varying degrees of disjunction.

The illustrations and descriptions presented herein, considered in relation to the observed facts as to chiasmata and construction of the spindle attachment, clearly show that the chromosomes of the complex of any species exhibit morphological distinctions. In 1903 Cannon (*5*) noted that the chromosomes of a hybrid having 26 pairs were ring-shaped at first metaphase but varied at first anaphase to U-, V-, and X-shaped. During diakinesis in *Gossypium barbadense*, Beal (*3*) observed O-, V-, X-, Y-, and 8-shaped pairs. In both American cultivated and Asiatic species, Baranov (*2*) apparently observed chromosome distinctions. An abstract of his article states:

> Clear differences were evident, chromosomes with equal and unequal arms, divided and undivided arms and with or without satellites being observed, as well as differences in size of the respective arms of the satellites.

Although the writer has not studied the somatic chromosomes, judging from the meiotic conditions it appears that they must be nearly equivalent in size. The majority of them are probably V-shaped and possibly exhibit the variations recorded by Baranov.

MEIOTIC BEHAVIOR

It is shown in the foregoing section that the majority of cultivated cottons occasionally exhibit irregularities during meiosis. The wild American species and *Gossypium sturtii* are shown to exhibit fairly normal meiotic behavior. Up to and through first anaphase the American cultivated species are shown to exhibit more irregularities than the Asiatic species. The irregularities in the former group involve quadrivalent formation, secondarily paired bivalents, and lagging bivalents; whereas those of the latter group involve only lagging bivalents. Also, in the latter case the percentage of lagging bivalents is less than in the former case. Following first anaphase, irregularities in the Asiatic species are shown to be more frequent than

in the cultivated American species.[6] It is found that in all cultivated species irregularities occurring during the first division occasionally result in granddaughter nuclei having less, and rarely more, than the normal haploid chromosome number. Finally the sporads of all cultivated species are shown to exhibit abnormalities in that they occasionally contain micronuclei and frequently microcytes.

Other authors have apparently observed such irregularities in cotton but have failed to recognize their importance. It is also very likely that irregularities have been observed in cotton which have been misinterpreted or attributed to poor fixation. Denham notes lagging bivalents in *Gossypium barbadense* (*10, p. 418, figs. 39–41*). He also occasionally observed "chromosome clumps" during diakinesis, which slightly reduced the total number of chromosomes. From his description and figures it appears that these "clumps" are probably quadrivalents. Although Beal (*3*) makes no statements regarding quadrivalent formation, his figures 24 and 25 clearly depict the occurrence of quadrivalents. The occurrence of 14 bivalents in an Asiatic-American hybrid, as observed by Longley (*20*), is evidence of the existence of quadrivalent affinity in the American parent. Secondarily paired bivalents have not previously been described or figured in the literature on cotton. However, Darlington points out that the latter phenomenon is generally disregarded as due to poor fixation. Longley (*20*) discusses abnormal cotton sporads. On the other hand, Banerji (*1*) states:

> Certain Indian cottons show a certain amount of sterility, but no irregularities in the reduction divisions were noted, except in the occurrence of a few sterile anthers.

Irregularities similar to those occurring in *Gossypium* are fairly common in plants. Lagging bivalents have been described and figured in *Viola striata* Ait. (*6*), *Spinacia oleracea* L. (*21*), *Prunus* hybrids (*7*), and in other plants. Darlington (*8*) attributes the condition to the degree of terminalization of chiasmata. The behavior at first anaphase in cultivated cottons supports Darlington's interpretations. At this stage, the majority of bivalents exhibit only terminal chiasmata and their partners appear to disjoin in normal fashion. The lagging bivalents, on the other hand, in nearly all cases exhibit 1 or 2 subterminal or interstitial chiasmata.

In cytological literature, the occurrence of quadrivalents and secondarily paired bivalents is mainly associated with either hybrid polyploidy or with self-polyploidy (*4, 9, 17, 23*). Longley (*20*) considers that the genetical behavior of the cultivated American cottons and their double chromosome number suggest that they are of tetraploid nature. Hence, the occurrence of quadrivalents and secondarily paired bivalents in these cottons supports Longley's suggestion, which is further supported by the similarity in shape and size of the bivalents. In figure 1 similar pairs are easily distinguishable.

The meiotic behavior of *Gossypium* hybrids (*5, 10, 20, 22*) clearly shows that the species within both the cultivated American and the Asiatic group are closely related. During meiosis in American-

[6] This reversal of behavior indicates that more of the lagging bivalent partners of the species having 26 pairs are distributed to daughter nuclei than in the case of such bivalent partners in the species having 13 pairs. The higher number of laggard partners distributed in the species having 26 pairs might be caused by the larger number of chromosomes involved, which may possibly delay the anaphase.

Asiatic hybrids, Nakatomi (*22*) reports 13 bivalents and 13 univalents, while Longley reports variable pairing, with as high as 14 bivalents. In the latter hybrids it remains to be determined whether the bivalent association is within the American haploid chromosome set or between the parental haploid chromosome sets. The answer to this question will show whether the American species having 26 pairs are of tetraploid nature, and also may be expected to throw light upon the degree of relationship between the Asiatic and American cultivated cottons. With these conditions unknown, it is impossible to give accurate phylogenetic interpretations.

POSSIBLE OCCURRENCE OF ANEUPLOID INDIVIDUALS

It has been shown that at least the male gametes of all cultivated cottons occasionally contain less and rarely more than the characteristic haploid chromosome number. Hence, the question arises why individuals are not found having somewhat more or somewhat less than the characteristic chromosome number. Such aneuploid individuals in cotton either have been overlooked or do not exist. If aneuploids do not exist in cotton, then it must be assumed that gametes containing more or less than the characteristic haploid chromosome number either degenerate or are incompatible. Since in the present study no degeneration was observed during pollen development, it is very unlikely that degeneration accounts for the lack of aneuploid individuals. No data are available concerning the incompatibility of such abnormal gametes in cotton. It is conceivable that they may be unable to cause fecundation, or that zygotes containing more or less than the characteristic chromosome number are unable to build a soma. However, the wide occurrence of aneuploidy in other genera (*Nicotiana*, *Datura*, *Matthiola*, *Solanum*, *Crepis*, etc.) leads the writer to believe that such individuals occur in cotton and simply have been overlooked or not recognized.

SUMMARY

The reported haploid chromosome numbers of 13 for all Asiatic cottons, for wild American species of *Gossypium*, and for *Gossypium sturtii*, and of 26 for all cultivated American cottons are confirmed. Of species hitherto unreported, *G. darwinii* is found to have 26 haploid chromosomes and *G. kirkii* approximately 24 diploid chromosomes.

Although a few bivalents in cultivated cottons are attached by interstitial chiasmata, the majority are attached by 1 or 2 terminal or subterminal chiasmata. The wild American species and *G. sturtii* apparently exhibit only terminal or subterminal chiasmata. The total number of attachments is relatively high for the small size of the chromosomes.

The wild American species and *G. sturtii* exhibit normal meiotic behavior.

Occasionally in cultivated American cottons from 1 to 5 quadrivalents are formed. Disjunction and distribution of partners in quadrivalents is apparently normal.

Apparently from 0 to 18 secondarily paired bivalents occur in the species having 26 pairs of chromosomes.

Occasionally, in all cultivated cottons during first anaphase, a few lagging bivalents occur. Generally the partners of such laggards finally disjoin and are distributed to opposite poles, or occasionally are left in the plasma. Rarely disjunction fails and the lagging bivalents are distributed as a whole to one pole.

Chromosome counts and behavior during the homotypic division show that spores of cultivated cottons occasionally have less and rarely more than the characteristic haploid number of chromosomes.

The sporads of the majority of cultivated cottons show more abnormalities than would be expected in the case of a species exhibiting normal meiotic behavior.

All pollen grains apparently develop normally.

Evidence is presented that the chromosomes within a cotton species are heterogeneous in shape and very nearly equivalent in size.

Evidence is presented that the species having 26 pairs are of tetraploid nature. However, attention is drawn to the fact that the final proof of this tetraploid nature and the consequent phylogenetic interpretations rest upon the type of pairing that occurs in American-Asiatic cotton hybrids.

The possible occurrence of aneuploids in cultivated cottons is discussed.

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DAMAGE TO PINUS MONTICOLA BY CRONARTIUM RIBICOLA AT GARIBALDI, BRITISH COLUMBIA[1]

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INTRODUCTION

In the fall of 1921 white pine blister rust, caused by *Cronartium ribicola* Fisch., was discovered in western North America.[2] [3] Extensive and intensive studies of the disease in this new range were begun in 1922. Scouting showed it to be present on western white pine (*Pinus monticola* Dougl.) in a number of widely scattered localities, principally in southwestern British Columbia.[2] [3] [4] The infection appears to have been introduced in 1910 on nursery stock of *P. strobus* L. shipped from France and planted near Vancouver. Aside from this, the earliest infection centers were of 1913 origin. These ranged from near Vancouver up to 130 miles northwest and north of that city. In 1922 considerable intensification of the rust, principally of 1917 and 1918 origin, was evident upon the pines, and a heavy wave of intensification of rust of 1920 origin also made its appearance.

It was evident from the beginning that in the smaller stock the rust would prevent the growth of merchantable timber crops of *Pinus monticola*, even when associated ribes [5] occurred scatteringly. Serious killing and injury were already in evidence in such stock close to susceptible ribes. By far the heaviest infection and most serious damage in any of the infection centers were found at Garibaldi,[6] British Columbia, about 60 miles north of Vancouver. To record the amount of this damage and to follow its intensification, three permanent plots were established.

DESCRIPTION OF PLOTS

The location of the plots established near Garibaldi is shown in figure 1. Plots 1 and 2 were laid out in the area of heaviest infection in 1922; plot 3 was laid out in 1923.

Plot 1 covered one-third of an acre and contained 178 trees of *Pinus monticola* ranging in height from 5 to 50 feet, but mainly 10 to 25 feet, and up to 9 inches in diameter at breast height, growing in moderate density under a light crown canopy of tall alders and poplars. The highly susceptible stink currant (*Ribes bracteosum* Dougl.) and prickly currant (*R. lacustre* (Pers.) Poir.) were present in profusion immediately under the trees and in an area of several acres bordering and surrounding the plot. It was estimated that an area of 965 square feet

[1] Received for publication Nov. 1, 1933; issued August 1934.

[2] DAVIDSON, A. T. WHITE PINE BLISTER RUST IN BRITISH COLUMBIA. (Report of Canadian conditions and work.) Third Western White Pine Blister Rust Conf. Rept. Proc. and Recommendations 3: 9-12. 1922. [Mimeographed.]

[3] LACHMUND, H. G. STUDIES OF WHITE PINE BLISTER RUST IN THE WEST. Jour. Forestry 24: 874-884. 1926. See p. 875.

[4] PENNINGTON, L. H. RELATION OF WEATHER CONDITIONS TO THE SPREAD OF WHITE PINE BLISTER RUST IN THE PACIFIC NORTHWEST. Jour. Agr. Research 30: 595. 1925.

[5] Plants of the genus *Ribes*.

[6] Formerly Daisy Lake, recently changed to Garibaldi.

on the plot was covered by the growth of these ribes in 1922. Counts and measurements of each species in 1923 gave a total of 205 plants of *R. bracteosum*, measuring in all 2,080 feet of live stem, and 180 plants of *R. lacustre* having a total of 679 feet of live stem. In this moist, moderately shaded natural inoculation chamber, conditions were ideal for infection, and the trees were almost completely covered

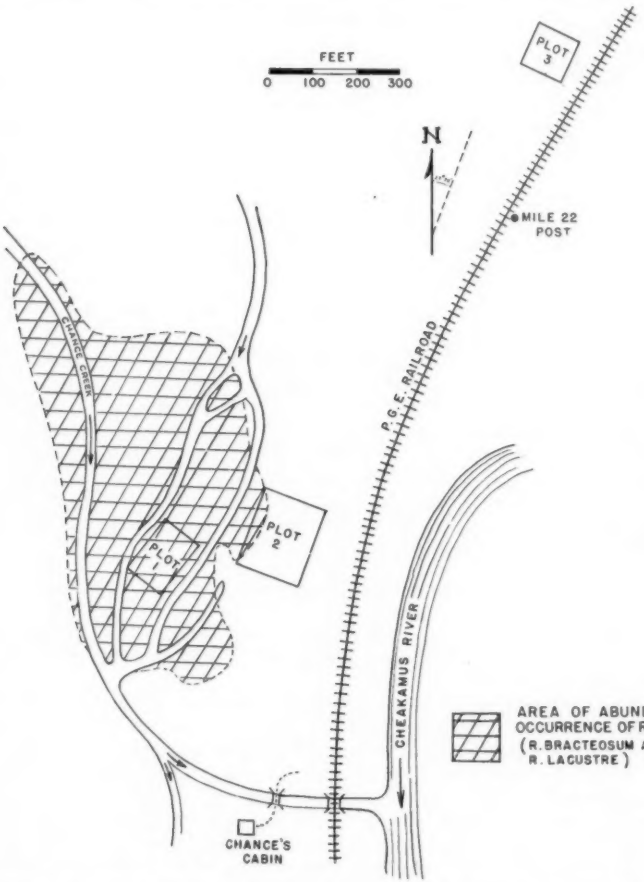

FIGURE 1.—Location of plots selected for study of damage to *Pinus monticola* by *Cronartium ribicola* at Garibaldi, British Columbia.

with cankers. By August 1922, over 40 percent of the trees in this plot had been killed by the rust.

Plot 2 covered two-thirds of an acre and bordered the area of heavy ribes growth. It contained 442 trees of *Pinus monticola* in an almost pure stand, ranging from saplings a few feet high to a few trees 13 to 20 inches in diameter at breast height and up to 95 feet high. The only ribes on the plot was *Ribes lacustre*, which covered an estimated

total area of 1,210 square feet close to the western border. Counts and measurements in 1923 gave a total of 93 plants and 863 feet of live stem of this currant. The rest of the plot was free from ribes. Over 90 percent of the trees were infected in 1922 but none had yet been killed. Decidedly the heaviest infection occurred on the western side of the plot, which bordered the same area of heavy ribes growth that surrounded and covered plot 1. Infection was relatively light on the side of the plot away from the ribes area. The largest trees showed little sign of damage, as seen from the ground, but upon close examination all of them were found to be heavily infected throughout the crown.

Plot 3 occupied one-fourth of an acre in an area of young mixed reproduction about 300 yards northeast of the area of the heavy growth of *Ribes bracteosum* and *R. lacustre* which covered plot 1 and bordered plot 2. Between the two areas was interposed an uneven and broken stand of mixed conifers and scattered hardwoods ranging largely from 60 to 70 feet in height. There were no ribes on this plot, but a few scattered bushes of *R. lacustre* and *R. sanguineum* Pursh (redflowering currant) grew within 30 yards of it. At the time of its establishment in 1923 this plot contained 194 pines, averaging about 12 feet and ranging in rapidly decreasing number from less than 5 to nearly 50 feet in height, together with other conifers and hardwoods (willows and poplars) of similar sizes. Only 40 percent of the pines were infected and the infection was light, often consisting of only 1 or 2 cankers to a tree.

METHODS

Each tree was tagged and numbered with a permanent aluminum label. In the fall of each year a record was made of the height and diameter of each tree, its condition, and the extent of infection and damage. On the more lightly infected smaller trees a fairly close count of the number of cankers was possible each year, but on the more heavily infected trees and on the larger trees, the number of cankers could only be estimated roughly in tens, hundreds, or thousands.

Counts and measurements of the number and size of the ribes on each plot were made each fall from 1923 to 1929, inclusive, with the exception of 1924, when the plants examined were so badly defoliated that no data could be obtained.

EFFECT OF INFECTION ON PINES

The extent of infection on plots 1 and 2 at the beginning of the study is indicated by the fact that for some of the larger and more heavily infected specimens the estimates of the number of cankers on one tree reached a maximum of 1,800 on plot 1 and 5,000 on plot 2.

The great majority of the cankers resulting from any given year of infection on *Pinus monticola* in the region under consideration make their appearance in the second year following infection.[7] [8] The general infection data from the plots and from the surrounding locality gave evidence that the original invasion of the rust in 1913 was followed by waves of heavy intensification in 1917, 1918, and 1920.

[7] LACHMUND, H. G. METHOD OF DETERMINING AGE OF BLISTER RUST INFECTION ON WESTERN WHITE PINE. Jour. Agr. Research 46: 675–693, illus. 1933.

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Another wave of intensification of the rust on the pines occurred in 1921, and enormous numbers of new cankers made their appearance upon the trees of plots 1 and 2 in 1923. Conditions for the general spread and development of the rust were not outstandingly favorable again until 1927. Owing to the abundance and the susceptibility of the ribes in the area, however, a large amount of intensification occurred on these plots each year. Intensification on plot 3 was relatively light.

TABLE 1.—*Yearly summary of damage to Pinus monticola caused by Cronartium ribicola on plots at Garibaldi, British Columbia*

PLOT 1

Year	Total trees—						Height of trees killed by rust		
	On plot	Used as basis[1]	Infected		Killed by rust		Minimum	Maximum	Average
	Number	*Number*	*Number*	*Percent*	*Number*	*Percent*	*Feet*	*Feet*	*Feet*
1922	178	172	172	100.0	71	41.3	7.0	30.0	15.9
1923	178	175	175	100.0	127	72.6	5.0	32.0	17.8
1924	178	175	175	100.0	158	90.3	5.0	35.0	20.6
1925	178	175	175	100.0	168	96.0	5.0	45.0	19.7
1926	178	175	175	100.0	169	96.6	5.0	45.0	19.9
1927	178	175	175	100.0	171	97.7	5.0	45.0	19.5
1928	178	175	175	100.0	172	98.3	5.0	45.0	19.5
1929	178	175	175	100.0	174	99.6	5.0	52.4	19.7
1930[2]	178								
1931	178	175	175	100.0	174	99.6	5.0	52.4	19.7

PLOT 2

1922	442	407	375	92.1	0	0.0	0.0	0.0	0.0
1923	442	419	398	95.0	10	2.4	1.0	10.0	4.9
1924	442	414	404	97.6	31	7.5	1.0	35.0	9.7
1925	442	409	406	99.3	72	17.6	1.0	35.0	10.7
1926	442	410	409	99.8	102	24.9	1.0	41.0	11.9
1927	442	410	409	99.8	137	33.4	1.0	50.0	13.9
1928	442	410	409	99.8	173	42.2	1.0	50.0	15.7
1929	442	396	395	99.8	221	55.8	1.0	58.9	17.0
1930	442	392	391	99.8	247	63.0	1.0	64.0	18.1
1931	442	389	389	99.7	255	65.8	1.0	74.4	19.5

PLOT 3

1923	190	189	76	40.2	0	0.0	0.0	0.0	0.0
1924	190	189	104	55.0	0	.0	.0	.0	.0
1925	190	187	135	72.2	2	1.1	1.1	1.2	1.2
1926	[3] 193	188	142	75.5	4	2.1	1.1	4.6	2.6
1927	[3] 194	186	141	75.8	5	2.7	1.1	4.6	2.9
1928	194	185	144	77.8	8	4.3	1.1	4.6	2.5
1929	194	185	144	77.8	11	6.0	1.1	14.6	3.5
1930	194	184	156	84.8	15	8.2	1.1	14.6	3.6
1931	194	183	157	85.8	21	11.5	1.1	14.6	4.1

[1] Trees killed through any agency other than rust (such as suppression) were not included.
[2] No data were taken during 1930.
[3] Small trees previously overlooked were added.

The time and extent of the damage resulting from the foregoing infection waves are given in table 1. By the fall of 1924, 11 years after the rust first entered the locality, over 90 percent of the trees on plot 1 had been killed. The killed trees ranged up to 35 feet in height and averaged over 20 feet. A few survivors persisted until 1929, when only a single relatively resistant individual, a tree about 40 feet high, remained alive. Resistance to the rust in *Pinus monti-*

cola is so unusual that a careful watch was subsequently kept of this specimen. In 1931 it was still alive but in poor condition, having about 175 cankers and a considerable number of branches that had been partly or entirely killed.

On plot 1, death of the trees resulted almost entirely from the killing of individual branches by innumerable twig and branch infections. Trunk infection played a minor role, killing 1 or 2 trees which ranged between 45 and 55 feet in height. Infection of the pines was uniformly distributed over the entire plot, conforming to the distribution of the ribes.

The data for plot 2 show that in 1922, although more than 90 percent of the pines were infected, none had been killed. In 1923 over 2 percent of the trees were dead; the average height of these was about 5 feet. From 1923 the percentage of trees killed rose steadily, and in 1931 had reached nearly 66 percent; the average height of the trees killed rose to more than 19 feet. From 1927 the killing included some trees ranging from 50 to 74 feet in height. Although all the larger trees were climbed and closely examined in 1928 and were found to have cankers which had entered or would enter the trunk so far down as to cause serious injury or death, and although it was estimated that these results would occur within 5 to 10 years, practically all the deaths on this plot resulted from the multiple killing of the individual branches, as on plot 1. In 1931 the great majority of the remaining trees were in a sickly condition, and it was apparent that few of them could survive longer than a few years more. These sickly trees included most of the largest of the plot, ranging up to 95 feet in height and over 12 inches in diameter at breast height.

On plot 3 in 1923 only about 40 percent of the trees were infected and none had been killed. In 1925, 72 percent were infected and about 1 percent had been killed. From then until 1931, the percentage of trees infected rose to about 86 percent and the percentage of trees killed to about 11 percent, the dead trees ranging to about 15 feet in height and averaging about 4 feet. The much lighter degree and slower progress of infection on this plot is in decided contrast to the extremely heavy infection and rapid damage on plots 1 and 2.

Plot 3, as already stated, was about 300 yards away from the heavy ribes concentration surrounding and bordering plots 1 and 2. The relatively light infection on plot 3 affords a good illustration of the rapid rate at which infection decreases with increasing distance from ribes, particularly when it is remembered that a considerable proportion of the infection on this plot undoubtedly came from the scattered ribes near it and in the territory between it and the heavy ribes concentration. If this amount of infection could be determined and subtracted from the total on the plot, the infection figures for this plot would unquestionably provide an even more striking contrast to those for plots 1 and 2.

Table 2 shows the rate of killing of the trees on plots 1 to 3 by height classes. The data for plots 1 and 2 indicate that where *Pinus monticola* is closely associated with an abundant growth of susceptible ribes the smaller trees are killed out almost completely within a very short time, and that even in trees as high as 60 to 70 feet the damage progresses rapidly. On plot 1, practically all the trees in the height

classes ranging to 30 feet had been killed by 1924, 11 years after the rust first entered the locality. In similar classes on plot 2, the majority of the trees had been killed by 1931, 18 years after the entrance of the rust into the locality. The much slower killing on plot 2 may be taken as further evidence of the rapid reduction of the intensity of infection with increasing distance from the ribes. The most rapid killing on this plot took place in the trees on the west side of the plot immediately adjacent to the heavy ribes growth.

TABLE 2.—*Rate of killing in different height classes of Pinus monticola by Cronartium ribicola on plots at Garibaldi, British Columbia*

PLOT 1

Height class (feet)	Tree basis		Trees killed by autumn of—										Total trees killed	Trees living in 1931	
	Number	Average height	1922	1923	1924	1925	1926	1927	1928	1929	1930	1931			
		Feet	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*Pct.*	*No.*	*No.*	*Pct.*
0.1–5.0	1	5.0	0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	1	0	0.0
5.1–10.0	14	8.4	78.6	92.9	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	14	0	.0
10.1–15.0	34	13.5	67.6	82.4	91.2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	34	0	.0
15.1–20.0	53	17.5	41.5	83.0	94.3	96.2	96.2	96.2	98.1	100.0	100.0	100.0	53	0	.0
20.1–30.0	60	24.9	23.3	61.7	91.7	98.3	98.3	100.0	100.0	100.0	100.0	100.0	60	0	.0
30.1–40.0	11	33.4	.0	36.4	63.6	72.7	81.8	90.9	90.9	90.9	90.9	90.9	10	1	9.1
40.1–50.0	2	47.5	.0	.0	.0	50.0	50.0	50.0	50.0	100.0	100.0	100.0	2	0	.0
Total	175	19.8	[1] 40.0	72.6	90.3	96.0	96.6	97.7	98.3	99.4	99.4	99.4	174	1	.6

PLOT 2

0.1–5.0	33	3.6	0.0	15.2	36.4	66.7	72.7	84.8	84.8	90.9	90.9	90.9	30	3	9.1
5.1–10.0	61	8.1	.0	6.6	16.4	41.0	49.2	62.3	67.2	73.8	82.0	85.2	52	9	14.8
10.1–15.0	55	13.2	.0	.0	9.1	25.5	41.8	50.9	63.6	74.5	80.0	81.8	45	10	18.2
15.1–20.0	44	17.9	.0	.0	4.5	11.4	25.0	31.8	52.3	70.5	72.7	72.7	32	12	27.3
20.1–30.0	73	25.0	.0	.0	1.4	4.1	12.3	24.7	38.4	53.4	63.0	65.8	48	25	34.2
30.1–40.0	45	35.1	.0	.0	2.2	6.7	8.9	17.8	28.9	40.0	48.9	57.8	26	19	42.2
40.1–50.0	34	46.4	.0	.0	.0	.0	.0	2.9	8.8	26.5	35.3	44.1	15	19	55.9
50.1–60.0	15	56.3	.0	.0	.0	.0	.0	.0	.0	20.0	33.3	33.3	5	10	66.7
60.1–70.0	18	67.0	.0	.0	.0	.0	.0	.0	.0	.0	5.6	11.1	2	16	88.9
70.1–80.0	7	75.5	.0	.0	.0	.0	.0	.0	.0	.0	.0	.0	0	7	100.0
80.1–90.0	2	84.8	.0	.0	.0	.0	.0	.0	.0	.0	.0	.0	0	2	100.0
90.1–100.0	2	91.5	.0	.0	.0	.0	.0	.0	.0	.0	.0	.0	0	2	100.0
Total	389	25.8	.0	2.3	8.0	18.5	26.0	34.7	44.0	55.5	62.2	65.6	255	134	34.4

PLOT 3

0.1–5.0	64	3.1	.....	0.0	0.0	3.1	6.3	7.8	12.5	15.6	18.8	25.0	16	48	75.0
5.1–10.0	42	7.4	.....	.0	.0	.0	.0	.0	.0	.0	4.8	9.5	4	38	90.5
10.1–15.0	18	12.9	.....	.0	.0	.0	.0	.0	.0	5.6	5.6	5.6	1	17	94.4
15.1–20.0	20	17.0	.....	.0	.0	.0	.0	.0	.0	.0	.0	.0	0	20	100.0
20.1–30.0	17	23.6	.....	.0	.0	.0	.0	.0	.0	.0	.0	.0	0	17	100.0
30.1–40.0	15	32.5	.....	.0	.0	.0	.0	.0	.0	.0	.0	.0	0	15	100.0
40.1–50.0	7	44.5	.....	.0	.0	.0	.0	.0	.0	.0	.0	.0	0	7	100.0
Total	183	12.5	.....	.0	.0	1.1	2.2	2.7	4.4	6.0	8.2	11.5	21	162	88.5

[1] 37.1 percent were killed before the autumn of 1922.

In the trees above 30 feet and ranging to 60 feet in height, the data from plot 2 show that from 33 percent in the 50.1–60.0-foot height class to 58 percent in the 30.1–40.0-foot height class were dead in 1931, and it could be estimated that the majority of the remaining trees on the plot, even in the largest size classes, would succumb in only a few years more.

The data from plot 3 show that in the trees farther removed from the source of sporidia, infection and killing occurred much more slowly. Only about 11 percent of the trees had been killed by the autumn of 1931, and the killing was primarily confined to the 0.1–5.0-foot height class, in which 25 percent were dead. The percentages of trees killed in the 5.1–10.0-foot and 10.1–15.0-foot height classes at this time, 18 years after the rust entered the locality, were only about 10 and 6, respectively, and none of the larger trees had yet been killed. Under these conditions of relatively light infection, killing and serious damage occur primarily by spread of the infection down the branches to the trunk and by the girdling of the latter. This is the general mode of damage in *Pinus monticola* [9] [10] and requires a considerably longer period, particularly in the larger trees, than when death results from the multiple infection and killing of individual twigs and branches, as on plots 1 and 2. The latter type of killing occurs only in the immediate vicinity of large concentrations of ribes or considerable numbers of the most susceptible ribes.

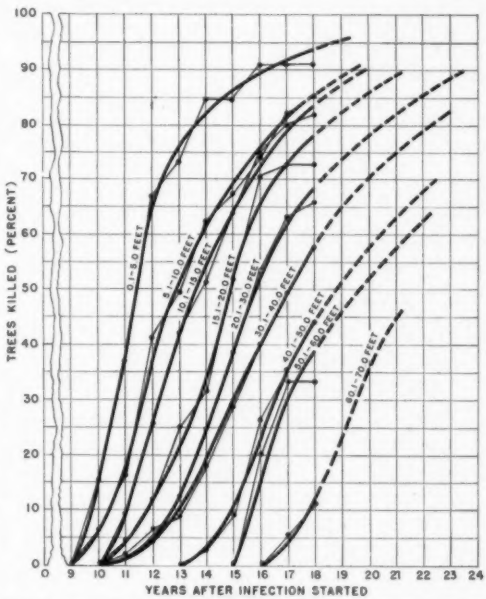

FIGURE 2.—Rate of killing of *Pinus monticola* by *Cronartium ribicola*, by height classes, on plot 2 at Garibaldi, British Columbia.

The data on rate of killing in the different height classes on plots 1 and 2 (table 2) provide the basis for some fairly definite estimates of the time required for killing under similar conditions in still larger height classes. The relatively small number of height classes on plot 1 and the degree to which killing had progressed in practically all classes at the time the plot was established permit only limited calculations. Plot 2, however, provides the basis for a fairly concrete synthesis of relationships in figures 2 and 3.

The percentage values of table 2 were first plotted and used for the development of the series of smoothed curves shown in figure 2 for percentage of trees killed over a number of years since infection first entered the locality, i.e., since 1913, for each height class. These curves showed a marked tendency to harmonize naturally with one another through most of their length, and this tendency was followed

[9] LACHMUND, H. G. See p. 883 of the reference cited in footnote 3.

[10] ——— GROWTH AND INJURIOUS EFFECTS OF CRONARTIUM RIBICOLA CANKERS ON PINUS MONTICOLA. Jour. Agr. Research 48: 475–503, illus. 1934.

as far as possible in the drawing of the curves and used as a governing factor in the determination of the direction of their extensions.

From the curves of figure 2 were read the values for each height class in number of years (from the original entrance of the rust) required for varying degrees of killing, at 10-percent intervals from 10 to 90 percent. These values were plotted and used as the basis for the development of the series of smoothed and partly harmonized curves in figure 3, giving time required for these different degrees of killing for different tree heights. The extensions of these latter curves indicate the time that probably would be required for the different degrees of killing in the height classes beyond those represented on the plot.

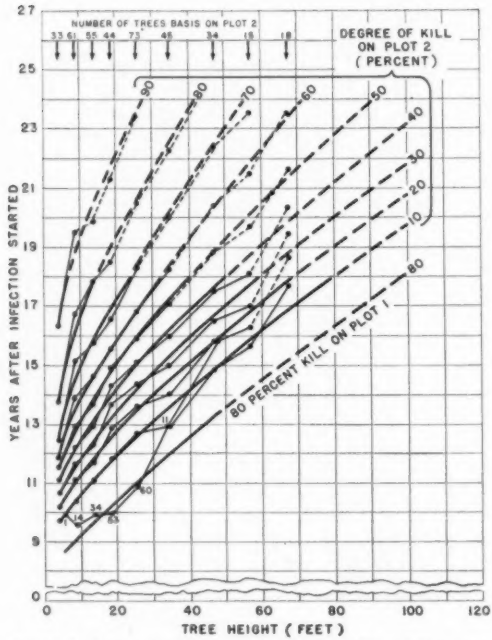

FIGURE 3.—Time required for various degrees of killing of *Pinus monticola* by *Cronartium ribicola* on plots 1 and 2 at Garibaldi, British Columbia.

Since the points for both figures permit the drawing of fairly symmetrical harmonic curves with little deviation from the points, it is believed that, up to certain limits which are discussed later, the curve extensions give values closely approximating the actual values.

For comparison of the effect of the infection on plot 2 with that on plot 1, values were read directly from table 2 to construct a curve in figure 3 for 80-percent kill on plot 1. The curve through the points so obtained was made to harmonize in form with the curves of plot 2 and is not in exact conformity to the direction indicated by the points, although the latter are all fairly close to the curve. The direction of the extension of this curve, therefore, is based entirely upon indications from plot 2.

Owing to changes in the form of the crown, which becomes narrower in relation to height in mature trees, it is questionable how much reliance can be placed on the curve-extension values beyond a height of about 100 feet. Furthermore, any great extension would be considerably beyond a safe margin from the actual data. Consequently the curves of figure 3 have not been extended beyond a tree height of 100 feet nor beyond 24 years.

The last examination of the remaining trees on the plots indicated that in all trees death would result within relatively few years. It seemed that all the largest trees, including those over 70 or 80 feet in height, as a group, had reached a sickly condition and would be likely to succumb to the disease more or less simultaneously in a few years. This would seem to indicate at least that the curve-extension values in figure 3 are not too low.

The relationships in figure 3 are self-evident. Where the tree height is 5 feet, the average time required after the entrance of infection to the locality, for the various degrees of killing under the conditions on plot 2, ranges from about 10 years for a 10-percent kill to about 15 years for an 80-percent kill. For a tree height of 40 feet, the range in time required for the same range in percentage of killing is about 14 to 24 years. It will be noted that even in the mature trees of the 100-foot height class, heavy loss (40-percent kill) is indicated within the relatively short period of approximately 23 years.

A comparison of the curve of plot 1 with those of plot 2 brings out in striking contrast the still greater rapidity of damage under the heavier infection conditions on plot 1. At a tree height of 5 feet, for example, the indicated time requirement on plot 2 is about 10 years for a 10-percent kill, whereas on plot 1 it is only about 9 years for an 80-percent kill. For the same degree of kill on plot 2 the period is approximately 14 years. Eighteen years is the indicated time required for an 80-percent kill at a tree height of 100 feet on plot 1 as compared with 23 years for a 40-percent kill on plot 2. However, owing to the method of constructing the curve for plot 1, the curve-extension values are less certain for this plot than for plot 2. The curve extension for plot 1 was swung upward a little less rapidly than is indicated by the data in order to harmonize it with the curves of plot 2. Therefore the plot 1 values for these larger trees are perhaps somewhat lower than they should be. In any event the difference between the values for the two plots is marked. The plot 1 values may be considered as indicative of the most extreme conditions of infection. Plot 2 probably represents approximately the minimum in degree of infection at which extensive killing occurs of the type represented on plots 1 and 2, i.e., by multiple killing of individual twigs and branches.

EFFECT OF INFECTION ON RIBES

As previously stated, plot 1 was located in the area of heavy growth of *Ribes bracteosum* and *R. lacustre*, plot 2 had a quantity of *R. lacustre* within its western border, and plot 3 was devoid of ribes. The yearly data on number of bushes and feet of live stem of the ribes on plots 1 and 2 (table 3) show that ribes were reduced but little, even by the heaviest natural infection.

On plot 1 examination of the pines in 1922 showed that a heavy production of aeciospores had commenced about 1920. Although, as shown in tables 1 and 2, a portion of the trees on this plot were already dead in 1922, a very heavy production of spores took place from trees remaining alive in that year and again in 1923; after 1923 the production of spores diminished rapidly because of the death of the trees, and after 1925 it was relatively light. However, there still continued

to be an enormous number of spores produced on the larger trees on the area nearby, which insured a heavy initial infection of the ribes each year.

TABLE 3.—*Effect of infection by Cronartium ribicola on ribes on plots 1 and 2 at Garibaldi, British Columbia*

Date [1]	Plot 1				Plot 2, *R. lacustre*	
	R. bracteosum		*R. lacustre*		Plants	Live stem
	Plants	Live stem	Plants	Live stem		
	Number	*Feet*	*Number*	*Feet*	*Number*	*Feet*
August 1923	205	2,080	181	679	93	863
October 1925	223	1,797	181	535	197	844
September 1926	403	1,920	237	652	289	863
October 1927	293	1,303	169	465	192	603
August 1928	424	1,769	155	434	286	749
September 1929	251	1,250	108	600	313	876

[1] No data were taken during 1924.

General infection data for the locality, including the plots, showed a particularly heavy infection and uredinial intensification of the rust on the ribes in 1923, and when data were taken on the number of bushes and footage of live stem in the fall of that year, many of the bushes showed branches which had been killed back by premature defoliation caused by the infection. Still further killing of live stem by the rust occurred in 1924 and 1925, as shown by the values on live-stem footage for 1925 in table 3.

In 1926 there was an increase in number of bushes and in footage of live stem. The new plants in most cases were sprouts from roots. In this year infection was relatively light, and growth more than balanced die-back on the older established plants. This, combined with a slight addition in footage provided by the new plants, accounts for the slight increase in total footage in this year.

Table 3 shows a large reduction in live-stem footage as well as in number of plants in 1927. In this year weather conditions were exceptionably favorable for ribes infection, much die-back resulted, and most of the new plants were killed. In 1928, infection was not so heavy and the ribes again increased. By this time, however, shading resulting from the increase in density of the crown canopy of the overstory of alders had so intensified that it was exerting a decidedly weakening effect upon the plants. The large reduction in live stem of *Ribes bracteosum* in 1929 is explained by the fact that some of the larger, older bushes were finally killed out completely, but this killing seems to have been caused as much by suppression as by the infection. *R. lacustre* appeared to be more tolerant of shade and increased its live-stem footage in this year.

Except that the live-stem total was maintained to a greater degree, the *Ribes lacustre* followed roughly the same course of development on plot 2 as on plot 1. The plants here were more in the open. For several years, however, the plants on plot 2 had been subjected to a heavier shower of aeciospores than those on plot 1 because of the death of the trees on the latter and the increase in sporulation on the trees

of the former. Thus, while a greater die-back resulted from the heavier production of aeciospores on plot 2 during the later years, this loss was counterbalanced by the greater number of new plants that became established as a result of the more open conditions obtaining there.

The data in table 3, taken generally in combination with the data of tables 1 and 2, indicate that ribes are capable of surviving the heaviest natural infection. The ribes on or near the plots have already weathered the period of heaviest aeciospore production. Aeciospore production in the area is now definitely on the decline, and with the death of the remaining trees the ribes will be left unhampered to compete for the occupancy of the area with the other plants of the new and modified ecological formation which will follow the elimination of the pines.

SUMMARY

In order to study the progress of damage to *Pinus monticola* by *Cronartium ribicola* two plots were laid out in 1922 and one in 1923 at Garibaldi, British Columbia, which is one of the heaviest infection centers known in western North America and which was invaded by the rust about 1913. The first plot was situated in the center of a heavy concentration of susceptible ribes, the second bordered the concentration, and the third was about 300 yards away. The trees on the first plot ranged mainly from 15 to 30 feet in height; those on the second plot ranged up to 20 inches in diameter at breast height, and 95 feet in height; those on the third plot were about the same size as those on the first plot.

The infection on the first two plots was exceedingly heavy. On plot 1 about 40 percent of the trees had been killed when the study began, 90 percent had succumbed by 1924, and only one remained alive in 1931. On plot 2 about 66 percent of the trees had been killed by the autumn of 1931, and it was estimated that the remainder would succumb in a few years. The killing on both plots 1 and 2 was caused by innumerable twig and branch infections which killed out the crowns.

On plot 3 only 40 percent of the trees were infected and none had been killed in 1923; in 1931 only about 86 percent of the trees were infected, and 11 percent were dead. The relatively light incidence of disease illustrates the close relation of the intensity of pine infection to the distance from the source of sporidia on ribes. The killing occurred earliest and to the greatest degree in the smallest trees.

Data based on extended curves are given to indicate time requirements for degrees of killing and trees of size classes greater than those represented on the plots. The ribes growth was killed back to some extent during the period in which the infected trees immediately associated with it remained alive and produced aeciospores, but it began to increase again after the trees had died.

RELATION OF THE POTATO FLEA BEETLE TO COMMON SCAB INFECTION OF POTATOES[1]

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INTRODUCTION

Worm-track injury on potato tubers caused by the larvae of the potato flea beetle (*Epitrix cucumeris* Harr.) (*5*)[3] continues to be a serious problem to potato growers in the Greeley area of Colorado. Considerable damage is done to the foliage of the potato plant by the feeding of the adult beetles, but the damage to the tubers caused by the feeding of the larvae is of much more serious consequence. The infection of the worm tracks by common scab (*Actinomyces scabies* (Thax.) Güssow) is the most important phase of the tuber injury. Since the mechanics of this infection appears not to have been described, it is the purpose of this paper to show how the flea-beetle larvae may aid in scab infection of the potato tuber by carrying the scab organism from the soil to the tuber.

Studies of the life history and morphology of the potato flea beetle are of no great importance in solving this problem. Several investigators have described the life history quite fully. Hoerner and Gillette (*2*) made a study of the flea beetle in Colorado and described the damage done to plants and tubers. Johannsen (*3*), Webster (*8*), Britton (*1*), and Stewart (*7*) described certain types of flea-beetle injury. More recently Webster, Baker, and Hanson (*9*) in their work on worm-track injury on potato tubers reported the type called "pimply" potatoes and the "sliver" type as causing heavy damage in certain sections of Washington. MacMillan and Schaal (*6*) reported serious injury to tubers in Colorado resulting from infection of the worm tracks with the common scab fungus. However, none of these investigators mentioned the manner in which the worm tracks become infected. Leach (*4*) found that the seed-corn maggot (*Phorbia fusciceps* Zett.) is an important agent in the dissemination and hibernation of the potato blackleg organism (*Bacillus phytophthorus* Appel) and in the inoculation of its host. In a similar manner the flea beetle acts as an agent in scab infection of the potato tuber.

FIELD EXPERIMENTS

Measures for controlling the flea beetle are receiving considerable attention at the Colorado Potato Experiment Station at Greeley. Daniels,[4] working in the Greeley area, reports efficient control by the

[1] Received for publication Jan. 30, 1934; issued August 1934.

[2] The writer is indebted to L. B. Daniels, of the Department of Entomology and Zoology, State Agricultural College of Colorado, for suggestions relative to the handling of the insects, and to E. S. Schultz, of the Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, for suggestions and criticisms in the preparation of the manuscript.

[3] Reference is made by number (italic) to Literature Cited, p. 258.

[4] DANIELS, L. B. Unpublished data. Colo. Agr. Expt. Sta. 1933.

killing of the adults with dusts and sprays. However, as long as the larvae feed on the tubers the disfiguring worm tracks will continue to reduce the quality of the tubers.

Each season when the experimental plot at the potato experiment station at Greeley was dug, two types of worm-track injury were noted. The more common of these was the type described by MacMillan and Schaal (*6*) as being infected with common scab. Frequently worm tracks were observed that appeared as faint lines on the surface of the tubers. Cultures of this type of worm track proved the absence of scab. Often a tuber was found on which every worm track was infected with scab. This was especially true of lots that had received no seed treatment.

Ten hills of nearly mature potatoes were selected at random from each of two plots and carefully dug by hand. One plot had been grown from seed treated with 1:1,000 mercuric chloride for 1½ hours; the second plot had been grown from seed that received no treatment. The 10 hills from the treated lot contained 46 tubers, 28 of which showed serious worm-track injury; 51 tubers from the untreated lot showed worm-track injury of the "pimply" type.

All the tubers were taken to the laboratory and the worm tracks were cultured for scab, the method described by MacMillan and Schaal (*6*) being used. After the surface of a tuber was thoroughly washed the worm track was sterilized with hydrogen peroxide. The material to be cultured was then macerated with a scalpel, transferred to sterile water blanks, and from these to melted nutrient agar in Petri dishes. After 24 hours of incubation at 86° F. the dishes were examined, and then isolations were made. Of 31 worm tracks cultured from the untreated lot of tubers, 28 showed the scab organism. Of the 46 tubers from the treated lot 28 showed worm-track injury. Culture of these worm tracks showed scab present in 4 of the injured tubers.

As already mentioned, Leach (*4*) found that the seed-corn maggot carries the blackleg organism to potato seed pieces. It has been contended that the potato flea-beetle larvae carry both externally and internally the common scab organism from the soil to the tubers. The fact that there are fewer scab-infected worm tracks on tubers grown from treated seed than on those grown from untreated seed indicates a sterilizing effect on the soil by the chemicals used in the treatment.

LABORATORY EXPERIMENTS

CULTURE OF THE LARVAE

One hundred flea-beetle larvae were collected in the field by carefully removing them from tubers on which they were feeding. These tubers were grown from untreated seed. The larvae were placed in clean sterile sand and taken to the laboratory for further study.

After being carefully removed from the moist sand the larvae were divided into two groups of 50 each. Those in group 1 were disinfected with 1:1,000 mercuric chloride for 15 minutes, and were then washed through a series of sterile water baths. The larvae in group 2 were neither disinfected nor washed. The disinfected larvae were removed from the last water bath and transferred directly to marked areas on nutrient agar in Petri dishes. The larvae in group 2 were transferred directly from the sterile sand to marked areas on nutrient

agar, then immediately removed and reserved for further study. The Petri dishes were placed in the incubator and held at approximately 74° F. for 48 hours. At the end of that time the plates were examined.

All the larvae disinfected with mercuric chloride were dead; none had moved from the marked area on the agar; no bacterial or fungus growth developed from the dead larvae. After 3 days the dead larvae were mashed on the agar. Growth developed from each of the dead mashed larvae. Culture of these growths showed the presence of bacteria and the common scab fungus in 42 of the 50 larvae; this indicates that the larvae harbored the scab organism internally.

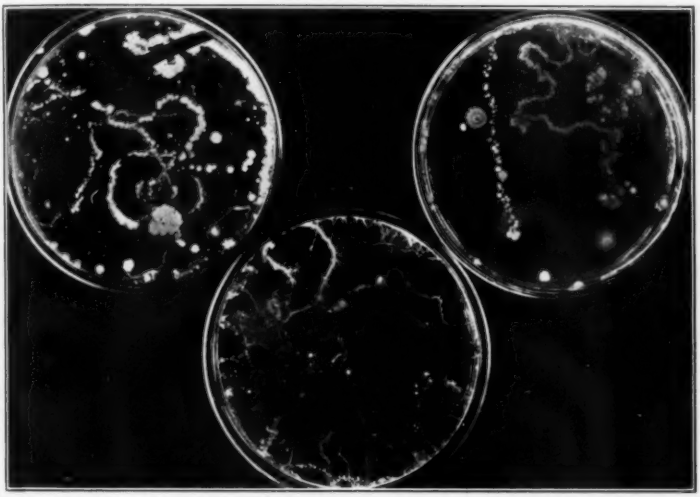

FIGURE 1.—Cultures showing development of bacteria and the scab organism (*Actinomyces scabies*) in the tracks made on nutrient agar by larvae of the potato flea beetle (*Epitrix cucumeris*).

Both fungi and bacteria developed on the agar where the unsterilized larvae had been placed. Culture of these growths showed the presence of the scab organism in 46 of the 50 larvae.

Of the 50 larvae retained from the untreated group 25 were washed through a series of 12 sterile water baths. After each washing the water adhering to the larvae was removed by placing the larvae on sterile filter paper. Twenty of the larvae survived these washings and when placed on nutrient agar in Petri dishes crawled about over the surface. The plates were placed in an incubator and kept there at 86° F. for 48 hours. Bacteria and the scab fungus developed on the agar where the larvae had crawled. Figure 1 shows the colonies of the scab fungus and bacteria growing from the tracks made on the agar by the larvae. Cultures of these colonies showed the scab organism to be present in the growths.

The remaining 25 larvae were placed on agar in Petri dishes without washing. They crawled about over the surface of the agar for several days. Fungus and bacterial growth developed and grew abundantly on the agar wherever the larvae had crawled. A larger

number of unidentified fungi grew from the tracks of these larvae than from those washed in sterile water.

CULTURE OF THE EGGS

A large number of adult potato flea beetles were collected in the field and taken to the laboratory, where they were caged in lamp chimneys used in collecting the eggs (fig. 2). Both the top and bottom of the chimney were covered with cheesecloth, the large end resting on blotting paper that covered a sand base. Previous to the introduction of the adult flea beetles the entire chimney cage and the sand base were sterilized with steam. A small piece of potato tuber was placed in the cage for the beetles to feed on. The sand and blotting paper were kept moist by adding sterile water to the sand at frequent intervals. Eggs were found on the blotting paper 3 days after the beetles were caged. The insects laid their eggs through the cheesecloth on the blotting paper, where they were readily picked up.

FIGURE 2.—Lamp-chimney cage used to collect eggs of the potato flea beetle.

Several preliminary experiments were conducted to determine a suitable disinfectant for the eggs; table 1 shows the results. A commercial compound, hexylresorcinol solution (S. T. 37), used in two different dilutions, was found to be unsatisfactory. Treating with mercuric chloride for 1 minute was the most effective method of disinfection; stronger dilutions prevented the eggs from hatching. The time element was an important factor. When the eggs were left in the mercuric chloride solution for periods longer than 1 minute, the number of eggs hatching was greatly reduced. After the eggs were removed from the disinfectant they were washed through six sterile water baths. When taken from the last water bath they were placed on marked areas in plates containing hardened nutrient agar. Several types of media were tried in the preliminary experiments; the nutrient agar was found best suited for the studies to be made. Culture of the growths arising from the eggs that did not prove to be surface-sterile showed that several species of bacteria were present, but they were not identified. No scab organisms were found in these growths.

TABLE 1.—*Results of culturing eggs of the potato flea beetle*

Disinfectant used	Period of treatment	Eggs treated	Eggs proved surface-sterile	Larvae emerging	Larvae from surface-sterile eggs showing no bacterial or fungus growth
		Number	*Number*	*Number*	*Number*
Mercuric chloride 1:1,000	2 minutes	50	50	4	4
	1 minute	50	47	20	20
	30 seconds	30	11	16	2
Hexylresorcinol solution (S.T. 37), commercial strength.		25	9	18	1
Hexylresorcinol solution (S.T. 37), 50 percent of commercial strength.		25	4	20	0

The larvae hatching from the surface-sterile eggs were picked up with a sterile needle and transferred to nutrient agar in Petri dishes. The larvae crawled about over the surface of the agar, but no bacteria or scab fungus developed on it.

Eggs that were transferred directly from the blotting paper to the nutrient agar showed growth of bacteria in every case. No scab fungus was found in the growths developing from the eggs.

GREENHOUSE EXPERIMENTS ON LARVAE AS CARRIERS OF THE SCAB FUNGUS

Larvae hatched from surface-sterile eggs under sterile cultural conditions were placed in soil close to small immature potato tubers growing in pots in the greenhouse in order to determine the relation of the larvae to dissemination of the scab organism. The soil had been sterilized with steam, and the seed had been treated with a 1:1,000 solution of mercuric chloride for 1½ hours before being planted. Two series of three pots each were used in this experiment and six flea-beetle larvae were placed in each of the six pots. Care was exercised in handling the sterile larvae, since they are easily killed. In series 2 the soil had been previously inoculated with cultures of common scab (*Actinomyces scabies*). In placing the larvae in the soil, the top inch of soil was removed, the larvae were placed about an inch from the growing tubers, and then the soil was replaced. The pots were set on the greenhouse bench and covered with cheesecloth cages. Soil moisture was maintained by adding sterile water frequently, for field observations have shown that the flea-beetle larvae prefer a soil with a relatively high moisture content.

TABLE 2.—*Results of placing surface-sterile flea-beetle larvae in uninoculated and in scab-inoculated soil containing growing potato tubers grown from disinfected seed*

[Six larvae were placed in each pot]

Series no.	Pot no.	Soil treatment	Tubers showing worm tracks	Presence of scab organism in worm tracks
			Number	
1	1	Sterilized; not inoculated	2	−
	2		3	−
	3		0	−
2	1	Sterilized; inoculated with scab organism	3	+
	2		2	+
	3		1	−

The plants were allowed to mature. The period between the introduction of the larvae and the maturity of the plants was 27 days. At the end of this period several small tubers were removed from each pot and the presence or absence of worm tracks noted. Table 2 shows the results of this experiment.

The tubers grown in the scab-inoculated soil (series 2) showed worm tracks that were rough and pimply (fig. 3), whereas those grown in the sterilized uninoculated soil (series 1) showed worm tracks that in most cases were but faint lines on the surface (fig. 4). Culture of the worm tracks on the tubers grown in the scab-inoculated soil (series 2) showed the presence of the scab fungus in 2 of the 3 pots. No scab was found in the worm tracks on the tubers grown in series 1.

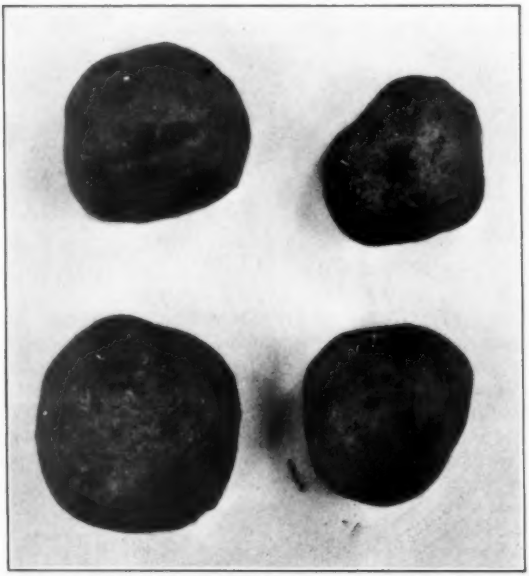

FIGURE 3.—Tubers grown from treated seed in soil inoculated with the scab organism (*Actinomyces scabies*). Surface-sterile flea-beetle larvae that had been placed in the soil made the worm tracks that became infected with the scab organism.

A second experiment involved the transfer of surface-sterile larvae from scab-inoculated soil to sterile soil. Six pots were used in 2 series of 3 pots each. The soil in each pot had been sterilized with steam previous to the planting of treated seed. The soil in the three pots in series 2 was inoculated with cultures of common scab before the seed was planted. Six sterile larvae were placed in the inoculated soil and allowed to remain there for 2 days. They were then carefully transferred to the uninoculated sterilized soil, where they were placed close to small growing tubers. These tubers were harvested 4 weeks after the larvae were placed in the soil. Several in each pot showed worm tracks. Of the 12 tubers taken from the three pots, 6 showed the rough, pimply type of worm-track injury. Cultures of the worm tracks showed the common scab organism to be present.

CONCLUSIONS

The potato flea beetle (*Epitrix cucumeris*) causes considerable damage to the potato plant by feeding of the adults on the foliage and the feeding of the larvae on the tubers. Investigators (*6*) have

shown that larval injuries to the tubers, commonly called worm tracks, become infected by the common scab fungus (*Actinomyces scabies*), thereby reducing the salability of the tubers. On tubers grown from untreated seed, scab lesions in the worm tracks were common, whereas on tubers grown from seed treated with a 1:1,000 solution of mercuric chloride for 1½ hours no worm tracks of the rough pimply type characteristic of scab infection appeared.

The scab organism is found in practically all soils in the potato-growing areas of Colorado. The worm-track injury offers a suitable point of attack for this organism. A part of the infection may result from direct contact with soil infested with the scab fungus. However, in carrying the scab organism, both internally and externally, the flea-beetle larvae serve as efficient agents of inoculation.

FIGURE 4.—Tubers grown from treated seed in sterilized soil. The worm tracks, made by surface-sterile flea-beetle larvae that had been placed in the soil, were not infected with the scab organism (*Actino mycescabiess*).

SUMMARY

The potato flea beetle (*Epitrix cucumeris* Harr.) continues to be a serious problem to potato growers in the Greeley area of Colorado. Worm tracks made on the tubers by the larvae become infected by the scab organism (*Actinomyces scabies* (Thax.) Güssow). This scab-infected type of worm track can be reduced by efficient seed treatment.

The flea-beetle larvae were found to carry the scab organism internally and externally under field conditions.

The scab organism was not found on the surface of the eggs of the potato flea beetle. Larvae hatching from disinfected eggs did not contain the scab organism, either internally or externally.

Worm tracks made by sterile larvae on potato tubers grown in sterilized soil did not become infected by the scab organism.

Larvae transferred from scab-inoculated soil to sterilized soil containing growing tubers made the scab-infected type of worm tracks on the tubers.

Potato flea-beetle larvae may cause scab infection of the potato tuber by carrying the scab organism from the soil to the tuber.

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RESULTS OF TRANSPLANTING GONADIAL TISSUE IN DAIRY COWS AND BULLS[1]

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INTRODUCTION

Gonadial tissue has been transplanted for the purpose of (1) making physiological and genetic studies, (2) relieving suffering (in human beings), and (3) rejuvenating and stimulating the genital organs. A great deal has been published concerning the transplanting of gonadial tissue in human beings, small laboratory animals, and birds, but few experiments have been reported with larger domestic animals, especially cattle. In the present work gonadial tissue was grafted on 11 cows and 5 bulls to determine whether the extra hormones would so stimulate, rejuvenate, or strengthen the genital organs that they would function normally. The work was done at the United States Dairy Experiment Station at Beltsville, Md.

REVIEW OF LITERATURE

Grunert [2] has reported the results of experiments with two cows, one of which was 15 years old and the other 12 years old. The 15-year-old cow had calved July 26, 1925; no oestrus was observed, and on June 21, 1926, ovarian tissue was grafted. Oestrus appeared August 8, the cow was bred and became pregnant. The 12-year-old cow had calved November 27, 1925; no ostrus was observed and ovarian tissue was grafted June 21, 1926. Oestrus was noted July 31, but the cow was not bred.

Haring [3] in reviewing his observations of grafting gonadial tissue in Switzerland stated that oestrus appeared in so-called sexual infantilism in heifers after grafting and also that libido appeared in bulls, but he made no mention of the effect on fertility.

Stäheli [4] grafted organs of young heifers onto 51 aged cows that had refused the bull for nearly a year. Forty-six came in season and mated. Of these, 31 conceived, and 15 were mated again and produced a second crop of calves.

Turner [5] reports that a successful autoplastic ovarian transplantation was made in a 6-month-old spayed heifer. At 12 months of age the heifer showed signs of heat, but the periods were not regular.

There is nothing in the literature to indicate that any irregularities of reproduction in cattle other than senility and infantilism have been corrected by grafting gonadial tissue.

[1] Received for publication Mar. 9, 1934; issued August, 1934.

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EXPERIMENTS WITH COWS

Grafts of ovarian tissue were made on 11 Jersey and Holstein Friesian cows in 1927 and 1928. In 4 of the cows grafts were made twice, at intervals of 7 days. The transplantations of ovarian tissue were made in the longissimus dorsi, serratus posticus, and trapezius muscles. The skin of the animal was cleaned of hair and dirt the day before the grafting. Vertical incisions were made through the skin in some of the animals and horizontal incisions in others. In the first four animals the tissue was implanted in the muscles and in all others it was attached to the roughened surface of the muscles. All operations were performed with the animals secured in the standing position. Chloral hydrate administered through the mouth served as a general sedative, and procaine was used as a local anaesthetic. Catgut sutures served to attach the gonadial tissue, and umbilical tape was used to close the skin incisons.

Ovaries were obtained from young healthy cows and healthy pigs. The cow ovaries were cut into 3 pieces, the larger pig ovaries were cut into 2 pieces, and the smaller ones were left whole. The ovarian tissue was kept in dry sterile glass containers until it was transplanted several hours later.

GENITAL HISTORY AND EXPERIMENTAL RESULTS

Table 1 shows the dates grafts were made on each cow, the source of the ovarian tissue, the number of pregnancies following the grafting, and the post-mortem appearance of the graft.

TABLE 1.—*Birth dates of the experimental animals, dates of grafting, sources of ovarian tissue, number of pregnancies following grafting, and post-mortem findings at grafted site*

Cow no.	Date of birth	Date of grafting	Pregnancy for which cow was being bred when grafted	Kind of ovarian tissue used	Pregnancies following grafting	Date of slaughter	Finding at site of grafting
626	July 11, 1924	Sept. 29, 1927	First	Cow	0	Jan. 27, 1928	Scar.
264	Dec. 20, 1920	do	Third	do	0	Feb. 10, 1928	Degenerating ovarian tissue.
462	Mar. 14, 1917	Oct. 25, 1927	Seventh	do	0	Jan. 24, 1928	Do.
490	Dec. 19, 1922	Nov. 1, 1927	Third	do	2	Sept. 30, 1930	Small scars.
253	Jan. 27, 1919	Jan. 13, 1928	Sixth	Pig	0	June 11, 1928	Scar.
279	Jan. 5, 1923	do Jan. 20, 1928	Fourth	do	0	Sept. 21, 1928	Do.
412 [1]	Dec. 30, 1918	Jan. 13, 1928 Jan. 20, 1928	Sixth	do	1	Feb. 20, 1930	Normal tissue.
442	Aug. 20, 1918	Jan. 13, 1928 Jan. 20, 1928	Seventh	do do	0	Dec. 6, 1928	Do.
468	Feb. 8, 1919	Jan. 13, 1928 Jan. 20, 1928	Fifth	do	0	Sept. 24, 1928	Scar.
613 [1]	Mar. 29, 1924	Jan. 13, 1928	Fourth	do	4	Jan. 6, 1933	Normal tissue.
609	Jan. 16, 1924	Jan. 20, 1928	do	do	1	Nov. 30, 1928	Scar.

[1] These cows were pregnant when subjected to the grafting operation and conceived later as indicated.

Cow 626

Cow 626, a registered Jersey, was 3 years 2½ months old when used in the grafting experiment in 1927.

This heifer had been mated 13 times, without conceiving, over a period of 16 months. During this time her genital organs appeared normal as determined by 15 examinations.

Grafting was done (Sept. 29, 1927) by separating the fibers of the left longissimus dorsi to form a pocketlike depression in which a piece of ovary was attached. In a similar manner two pieces of ovary were attached in a pocket made in the right trapezius cervicalis. The skin sutures were removed October 3, and the wounds were completely healed by October 29.

This animal did not conceive following the operation, although she was mated at four consecutive oestrus. She was slaughtered January 27, 1928, and the organs and tissues throughout the body appeared normal. Only dense scar tissue was found at the sites of the grafts.

Cow 264

Cow 264, a registered Holstein, was 6 years 9 months old when used in the grafting experiment in 1927.

This cow had been bred 11 times for her initial conception, giving birth to a normal calf July 1, 1924, after a gestation period of 293 days. Two matings were required for her second conception, which terminated in the birth of a normal calf on the two hundred and eighty-sixth day. Following this calving, 13 services between February 19, 1926, and August 21, 1927, inclusive, did not result in conception. On seven examinations the genital organs appeared normal, then on February 24, 1927, the uterus seemed to be enlarged as if she had been pregnant for approximately 40 days. Ten examinations between April 8 and September 28, 1927, inclusive, each revealed a soft uterus hanging forward in the abdominal cavity.

Ovarian tissue was grafted in this cow on the same day (Sept. 29, 1927) and in the same way as in heifer 626, except that a corpus luteum of the ovary was discarded. The skin sutures were removed October 3, and the wounds seemed completely healed October 29.

This animal showed no oestrus after the operation. The uterus showed little improvement as determined by five examinations, and the cow was slaughtered February 10, 1928. The walls of the uterus were found to be oedematous, with adhesions over the anterior parts of the uterus. The lymph glands around the pelvic inlet were enlarged.

Sections from the grafted area of the musculature showed small masses or islands of cells which were recognized as remnants of the ovarian graft. These masses of partially degenerated cells were surrounded by fibrous tissue proliferations and there appeared to be some slight degeneration of the adjacent muscle tissue. There was a noticeable round cell infiltration and also marked vascularization of the entire grafted area (fig. 1).

Cow 462

Cow 462 was a registered Jersey, 10 years 7 months old when used in the grafting experiment in 1927.

The portion of the breeding history of this cow on which there is a complete record is given here. She conceived in one mating, and on June 25, 1922, aborted after a 239-day gestation period. After this abortion she was bred seven times before she conceived, delivering a normal calf October 11, 1923, after a 272-day gestation period. During the next sexual season nine services were required before she conceived, delivering a normal calf June 28, 1925, after a 282-day

gestation period. She was than bred only three times before she conceived, delivering a normal calf August 13, 1926, after a 280-day gestation period. She was then mated eight times without conception.

One month after calving in August 1926 a cyst the size of a baseball was found on the right ovary and a smaller one on the left ovary. Cysts were present until June 1927, when they disappeared leaving the ovaries enlarged. Five examinations during July and early August revealed a soft toneless uterus and enlarged ovaries. During late August, September, and early October, five examinations revealed that the uterus was normal in size and consistency and that there was no change in the ovaries. Throughout the summer the vulva was constantly swollen to approximately the size of that of a cow ready to freshen.

Ovarian tissue was grafted in this animal October 25, 1927. An incision was made in the left longissimus dorsi and two pieces of ovary

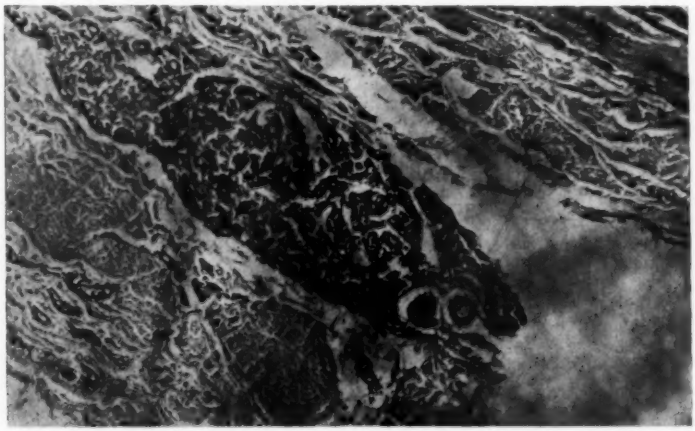

FIGURE 1.—Section from muscle of cow 264 showing remnant of ovarian graft. × 90.

were attached. Another piece was anchored to the roughened surface of the serratus posticus, and three pieces were anchored in a pocket in the right trapezius cervicalis. The skin sutures were removed November 1. At this time a hard swelling about 4 by 7 inches was noticed at the point of the operation on the back and a similar enlargement about 2 by 3 inches in size at the site of the operation on the neck. These enlargements broke November 13 and discharged a thick yellow pus. The wounds were healing normally by November 19. The swelling of the vulva had disappeared by November 1. When healing of the wounds assumed normal appearance the vulva began to enlarge again. There was no change in the condition of the rest of the genital organs. This animal was mated twice after the operation and did not conceive.

Autopsy on January 24, 1928, revealed enlarged dense ovaries with adhesions around them. The walls of the uterus were thick. Cysts were found in the walls of the vagina. Many minute blood vessels were observed in the region of the transplanted tissue. Tissue

resembling small ovarian follicles was found in the trapezius cervicalis and some dried tissue was noted in the region of grafting on the back.

Histologically the grafted area of the musculature showed a small portion of the transplanted ovarian tissue, surrounded by fibrous tissue. There was lymphocytic and round cell infiltration, and a number of multinucleated cells were noted. The grafted area showed extensive vascularization. Hemorrhages were observed toward the center of the area and also degenerated muscle tissue, masses of pus cells, and necrotic material (fig. 2).

Cow 490

Cow 490, a registered Jersey, was approximately 4 years 11 months old when used in the grafting experiment in 1927.

This cow had been bred three times before she conceived May 2, 1924. A nomal calf was born 278 days later. Following this calving

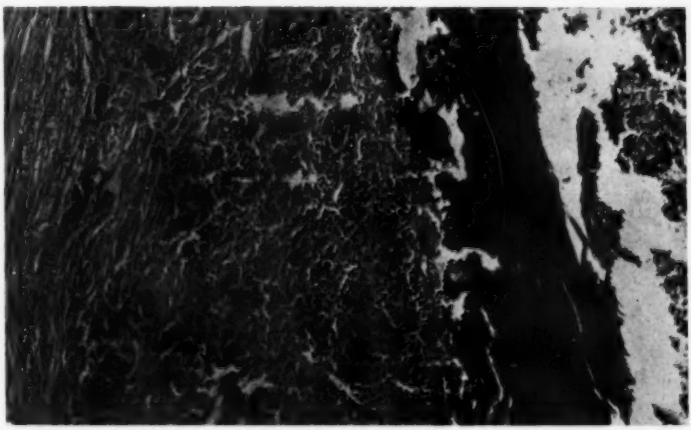

FIGURE 2.—Section from muscle of cow 462 showing remaining portion of transplanted ovarian tissue. × 90.

she conceived to the fifth service on October 5, 1925. She aborted 181 days later. Between the time of this abortion and the transplanting of ovarian tissue this cow was bred 15 times without conception. A metritis followed the abortion, and by April 1927 the ligaments supporting the genital organs had so relaxed that the uterus hung far forward in the abdominal cavity. By September 1927 there was much improvement in the tone of the uterus and the ligaments had strengthened sufficiently to hold the genital organs near their normal position, where they remained until after the grafts were made.

Grafting was done November 1, 1927. An incision was made in the right trapezius cervicalis and pieces of one ovary were attached to this cut surface. In the same manner pieces of another ovary were attached to a cut surface of the left longissimus dorsi. On November 2 both wounds were swollen. On November 8 diffuse swellings extended over the right side of the neck and over most of the left side of the back. The sutures were removed and the lower ends of the

wounds opened. A foul-smelling, brownish liquid ran from the openings. The wounds then healed slowly. In August 1928 an abscess formed at the site of grafting on the neck. It healed rapidly when drained.

The uterus became slightly relaxed a month after the grafting and remained so until conception took place at the sixth mating on October 5, 1928. The cow gave birth to a normal calf 277 days later. Only one service was required for the next conception, which terminated normally at the end of a 281-day gestation period on September 23, 1930.

On autopsy September 30, 1930, the uterus was found partly involuted. Only small scars were observed at the sites of the transplanted ovarian tissue.

Cow 253

Cow 253, a registered Holstein, was approximately 9 years old when used in the grafting experiment in 1928.

This cow had given birth to her first calf July 17, 1921. She then conceived to the second mating and calved normally at the close of a 281-day gestation March 19, 1923. Following this calving she conceived, to the sixth mating, on January 26, 1924, and aborted 270 days later. After this abortion she conceived, to the third mating, May 6, 1925, and a normal calf was born 287 days later. The next conception occurred, to the first mating, on June 22, 1926, and a dead calf was delivered 293 days later. Between the time of this calving and the transplanting of ovarian tissue this cow was bred nine times without conception. No abnormalities were found in the genital organs during this time.

Grafting was done on January 13, 1928. Three pieces of ovarian tissue were attached to the roughened surface of the right trapezius thoracalis, and one whole ovary was placed in a pocket in the lower part of the wound. The wound seemed to be clean and healing when the sutures were removed on January 23, but on January 30 a slight discharge of pus was noticed coming from the lower end of the wound and a piece of foul ovary was removed February 6. After its removal the wound healed rapidly.

Only one oestrus (May 1, 1928) was observed after ovarian tissue had been grafted in this cow. On autopsy (June 11, 1928) adhesions were found around the ovaries. Scar tissue only was found at the place of grafting.

Cow 279

Cow 279, a registered Holstein, was 5 years old when used in the grafting experiment in 1928. Conceiving on April 14, 1924, to the first mating, this cow gave birth to a dead calf 278 days later. Only one service (July 9, 1925) was needed for the second conception, which terminated in the birth of a normal calf 275 days later. She was bred only once (July 19, 1926) for the third conception, which terminated in an abortion 198 days later. Between this abortion and the time of grafting ovarian tissue 10 matings did not result in conception. This abortion was followed by metritis, and by December 1927 the ligaments supporting the genital organs had relaxed to such an extent that the uterus hung forward in the abdominal cavity.

Grafting was done on this cow at the same time (Jan. 13, 1928) and in the same manner as on cow 253. There was a little swelling around the wound January 16. The skin sutures were removed January 23 and complete healing had taken placed by February 7. Seven days after the first grafting three pieces of ovarian tissue and one whole ovary were grafted to the medial surface of the left trapezius thoracalis. The skin sutures were removed January 30 and the wound was completely healed by February 7.

This cow did not conceive at two matings following the operations. The uterus remained forward in the abdominal cavity until the animal was slaughtered September 21. On autopsy congestion around the pelvic inlet was noted. The walls of the uterus were thin and soft and the ovaries were enveloped in thick connective tissue. Only scar tissue was found at the sites of grafting.

Cow 412

Cow 412, a registered Jersey, was approximately 9 years old when used in the grafting experiment in 1928.

The early records of the activities of the genital organs of this cow are incomplete. She gave birth to normal calves October 21, 1923, November 26, 1924, February 14, 1926, and May 16, 1927. The genital organs seemed normal after the last calving. She was mated again on December 23, 1927.

Grafting was done at the same time (Jan. 13, 1928) and in the same manner as on cow 253. There was diffuse swelling over the grafted area on January 16, but it had practically disappeared by January 19. The skin sutures were removed January 23 and complete healing had taken place by February 7. Seven days after the first grafting three pieces of ovarian tissue were grafted to the roughened surface of the left trapezius thoracalis. The skin sutures were removed January 30 and the wound was completely healed by February 7.

An examination on February 14 showed that conception had occurred 21 days before the first grafting operation. This pregnancy terminated in the birth of a normal calf after a gestation period of 281 days. The next conception occurred from one mating on December 12, 1928, and a normal calf was born 285 days later. Three services following this calving did not result in conception. The genital organs seemed to be normal when examined during this sexual season.

On autopsy February 20, 1930, the walls of the uterus were found to be gelatinous and the fallopian tubes closed. Only connective tissue was found at the sites of grafting.

Cow 442

Cow 442, a registered Jersey, was approximately 9 years 5 months old when used in the grafting experiment in 1928.

The early records of the activities of the genital organs of this cow are incomplete. On December 3, 1920, she had aborted after a 224-day gestation period. Following this abortion she conceived to the first mating and gave birth to a normal calf February 1, 1922, after a 272-day gestation period. She then conceived to the second mating,

delivering a weak calf April 30, 1923, after a 273-day gestation period. Conception took place again when the cow was mated the second time. This pregnancy continued 261 days, terminating in the delivery of a normal calf. Following this calving she conceived to the fourth mating and aborted 201 days later on July 3, 1925. Then she conceived to the second mating and aborted 237 days later on July 26, 1926. She was then bred six times before the grafting operation, but did not conceive.

Involution of the uterus seemed complete 40 days after her abortion in 1926. By the end of another 30 days the uterus was large, its walls were thick, and there was a white discharge when the organ was massaged. This discharge continued until December 17, or approximately 5 months after the abortion. The uterus seemed to have returned to its normal nonpregnant size by March 3, 1927. It remained so until after the ovarian grafts were made. Breeding was not attempted until April 13, 1927, and six matings thereafter failed to produce conception.

The grafting of ovarian tissue was done (Jan. 13 and 20, 1928) in the same manner as described for cow 412. Healing was complete by February 7. An examination on February 14 revealed that the ligaments supporting the genital organs had relaxed to such an extent that the uterus hung forward in the abdominal cavity. The uterus remained in this position until August, when it assumed its normal position. On November 15 adhesions were found around the ovaries. The cow was mated twice after the grafting of ovarian tissue and did not conceive.

Autopsy on December 6 revealed adhesions around the ovaries and fallopian tubes. Only connective tissue was found at the points of grafting.

Cow 468

Cow 468, a registered Jersey, was approximately 8 years 11 months old when grafted with ovarian tissue in 1928.

The records of the early activities of the genital organs of this cow are incomplete. On January 5, 1924, she delivered a normal calf, after a 276-day gestation period. She then conceived to the fourth service and delivered a normal calf July 20, 1925, after a 283-day gestation period. The next conception occurred with only one mating, and a dead calf was born 277 days later, on September 1, 1926. Involution of the uterus seemed normal after this calving. Later the uterus enlarged and discharged pus. One year later it returned to its normal nonpregnant size. Between the 1926 calving and the death of the cow only one oestrus appeared. This was on March 8, 1927. After that the right ovary and fallopian tube became enlarged.

The grafting of ovarian tissue was done (Jan. 13 and 20, 1928) in the same manner as described for cow 412. Healing was complete by February 7. Following this there was no apparent change in the uterus and no oestrus occurred. The right ovary and fallopian tube were removed surgically March 17. Abscesses appeared in the udder, right flank, and the throat region in August.

Autopsy on September 24 revealed abscesses in the udder and under the skin in the throat region, and fibrous proliferations (fibroplastic peritonitis) were noted throughout the abdominal cavity and

over the genital organs. The fluids of the body cavities were gray in color. Only scar tissue was found at the sites of grafting.

Cow 613

Cow 613, a registered Jersey, was approximately 3 years 9 months old when used in the grafting experiment in 1928.

She conceived to her first mating, July 11, 1925, but the pregnancy terminated in an abortion 164 days later. Five matings then occurred before conception again took place, June 2, 1926. This pregnancy terminated in an abortion 264 days later. The genital organs seemed normal following the abortion. She was bred nine times, after which an examination of the semen of the bull showed inactive spermatozoa. The cow was then mated with a normal young bull on December 24, 1927.

Grafting was done (Jan. 13, 1928) in the same manner as described for cow 253. The skin sutures were removed January 23 and the wound was completely healed by February 7.

An examination on February 14 revealed that conception had taken place 20 days before the grafting operation. On October 4 a normal calf was born, after a 285-day gestation period. Four conceptions occurred after this calving. The first two required only one mating each. The third resulted from the ninth service, and the fourth required only one mating. The second pregnancy ended in the delivery of a dead calf, and the other pregnancies produced normal calves.

Autopsy January 6, 1933, showed the uterus in a normal involuting condition from the last calving on December 17, 1932. Only connective tissue was found at the site of the transplanted ovarian tissue.

Cow 609

Cow 609, a registered Jersey, was approximately 4 years old when used in the grafting experiment in 1928.

She had conceived to her first mating, April 23, 1925, but aborted 212 days later. Then conception resulted from one mating on February 4, 1926, and she aborted 120 days later. She was bred the third time October 18, 1926, but aborted 229 days later. She was then bred nine times but failed to conceive. No abnormalities were found in the genital organs during this time.

Grafting was done on January 20, 1928. The surface of the left trapezius thoracalis was roughened and five pieces of ovaries were attached. The skin sutures were removed January 30 and healing was complete by February 7.

The cow conceived at the sixth mating following the transplanting of ovarian tissue. A fetus was found in the uterus on autopsy November 30, 1928. The genital organs appeared normal. Only scar tissue was found at the site of the ovarian grafts.

EXPERIMENTS WITH BULLS

The technic of grafting testicular tissue in the five bulls differed but little from that of grafting ovarian tissue in the cows. Testicular tissue obtained from the testicles of young bulls and pigs was attached to the roughened surface of the trapezius muscle, and quinine urea

hydrochloride was used as a local anaesthetic. The testicles from the young bulls and pigs were used as soon as possible after they were collected. In the meantime they were kept in dry sterile glass containers.

SERVICE RECORD AND EXPERIMENTAL RESULTS

Table 2 shows the service records of the experimental bulls, when mated to fertile cows, before and after the implantation of testicular tissue.

TABLE 2.—*Service records of experimental bulls mated to fertile cows, before and after the bulls had received transplanted gonadial tissue*

Bull no.	Date of birth	Dates of grafting	Date of slaughter	Year	First quarter		Second quarter		Third quarter		Fourth quarter	
					Services	Conceptions produced	Services	Conceptions produced	Services	Conceptions produced	Services	Conceptions produced
1	July 29, 1917	Dec. 9, 1927 Jan. 23, 1928 May 3, 1928	Nov. 20, 1928	1926	10	5	13	4	22	3	15	4
				1927	14	3	30	7	36	1	14	0
				1928	3	0	1	0				
2	Apr. 30, 1915	Dec. 9, 1927 Jan. 23, 1928	Mar. 2, 1928	1926	13	3	10	4	12	5	12	4
				1927	9	2	1	1	13	0	21	0
				1928	3	0						
3	Apr. 21, 1911	Feb. 24, 1928	July 9, 1928	1926	3	1	2	2	3	0	5	3
				1927	7	1	3	2	2	1	5	1
				1928	7	0	1	0				
4	Aug. 18, 1921	....do....	Sept. 12, 1929	1926	18	6	8	5	4	3	10	5
				1927	10	3	15	6	14	4	18	4
				1928	17	4	22	9	7	1	10	1
				1929	16	5	5	3	4	0		
5 [1]	June 9, 1926	May 3, 1928	July 9, 1928									

[1] See discussion in text, p. 276.

BULL 1

Bull no. 1 was a registered Jersey born July 29, 1917.

The activity of the spermatozoa of this bull, as seen through the microscope, was never greater than 50 percent during 1926 and the first 9 months of 1927. No activity was found during the last 3 months of 1927 and during 1928. The spermatozoa stained poorly and many heads were without tails. In October 1928 only 25 percent appeared normal morphologically. Spermatozoa numbered from 1 million to 166 million in a cubic centimeter.

On December 9, 1927, two pieces of bull testicle, which retained the greater part of the epididymis, were fastened to the roughened surface of the left trapezius thoracalis. On December 12 a dark yellow discharge came from the lower part of the wound. The skin sutures were removed and the wound cleaned. On December 17 the wound was found gaping open. There appeared to be firm live testicular tissue in the upper part of it. The lower part of the incision held a small amount of debris, otherwise healing was progressing favorably. By January 13, 1928, the wound appeared to be healed.

No change was apparent in the usefulness of this bull following this grafting, but there was evidence of increased sexual desire. His passive disposition also changed to one of aggression.

On January 23, 1928, two pieces of testicle, about 2 inches in diameter, edges one-fourth inch thick with the parenchymatous tissue bulging to about one-half inch thickness, were attached to the

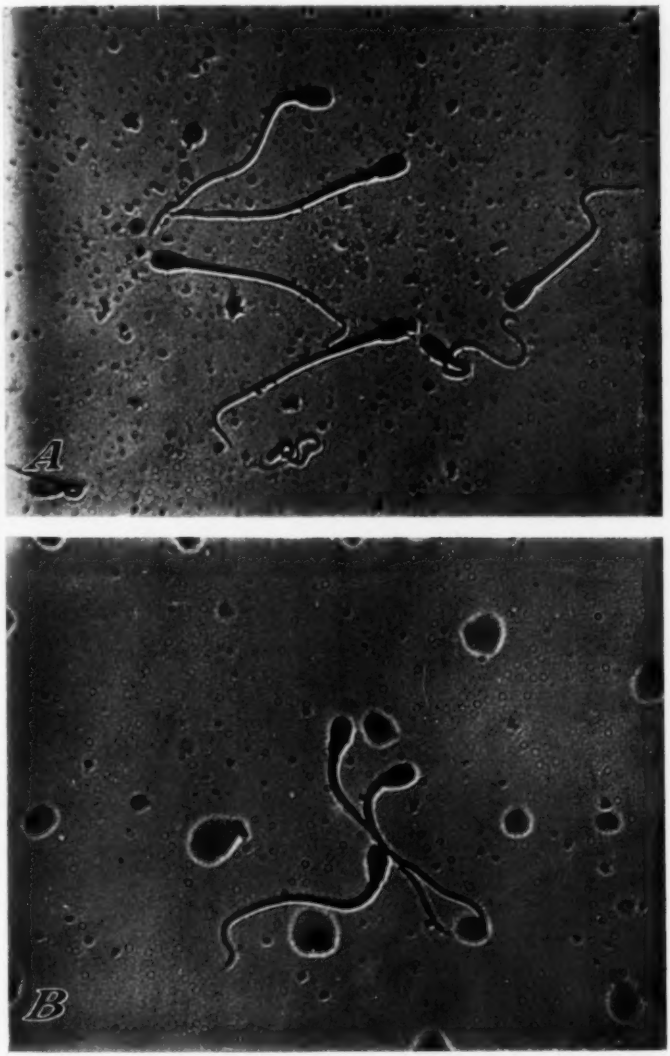

FIGURE 3.—Spermatozoa collected from bull no. 1: *A*, 27 days before grafting; *B*, 152 days after first grafting and 6 days after third grafting. × 460.

roughened surface of the right trapezius thoracalis. These pieces of testicle were as warm as the surrounding tissue where they were attached. The skin sutures were removed February 7 and the wound

seemed to be healing normally. On February 24 some dried tissue protruded through the skin and was removed. No change in the usefulness of the bull occurred.

On May 3, a third grafting was tried. Three pieces of pig testicle were attached to the roughened surface of the right trapezius thoracalis and six pieces were attached to the roughened surface of the left trapezius thoracalis. The skin sutures on the left side were removed May 14. The wound had a disagreeable odor but appeared dry and clean. The skin sutures on the right side were removed May 26. Both wounds healed by first intention.

The spermatozoa of this bull did not change in activity, staining ability, or morphology, following these graftings (fig. 3). No conception occurred from the use of this bull after September 1927.

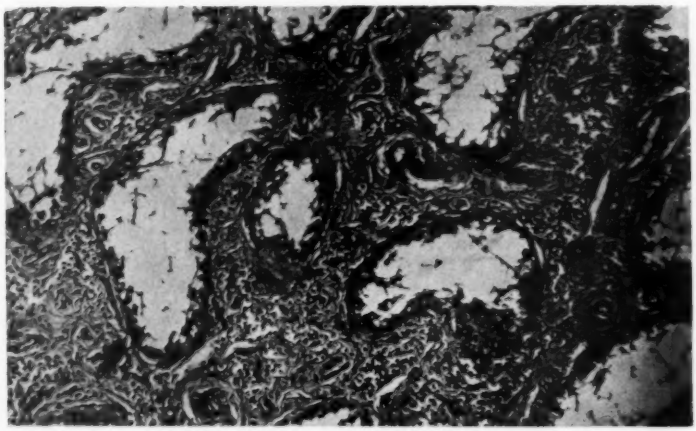

FIGURE 4.—Section from testicle of bull no. 1. × 90.

Following each operation increased appetite and aggressiveness were noted.

This bull was slaughtered November 20, 1928, and the lumbar vertebrae were found to be fused into one piece. The sixth and seventh thoracic vertebrae were fused with a bony growth extending into the thoracic cavity. The sixth, seventh, and eight ribs had sharp bony growths projecting into the thoracic cavity. Small abscesses were found where these sharp projections had injured the lungs. The genital organs were normal in shape but appeared atrophied. Scar tissue only was found at places of grafting.

Sections from the testes of this bull showed extensive atrophic changes throughout. There was almost a complete absence of tubular epithelium in the various sections examined, and in many of the tubules the epithelial structure had been replaced by concentric layers of connective tissue, which was gradually closing the lumen of the tubules. In some parts of the testes there was almost complete fibrosis, many of the tubules having been completely obliterated. A number of the remaining tubules contained deposits of a brownish pigment. Sections from the epididymis showed sloughing of the tubular epithelium and also extensive fibrous changes (fig. 4).

BULL 2

Bull no. 2 was a registered Jersey born April 30, 1915. Early in 1926 microscopical examinations of the semen of this bull revealed a

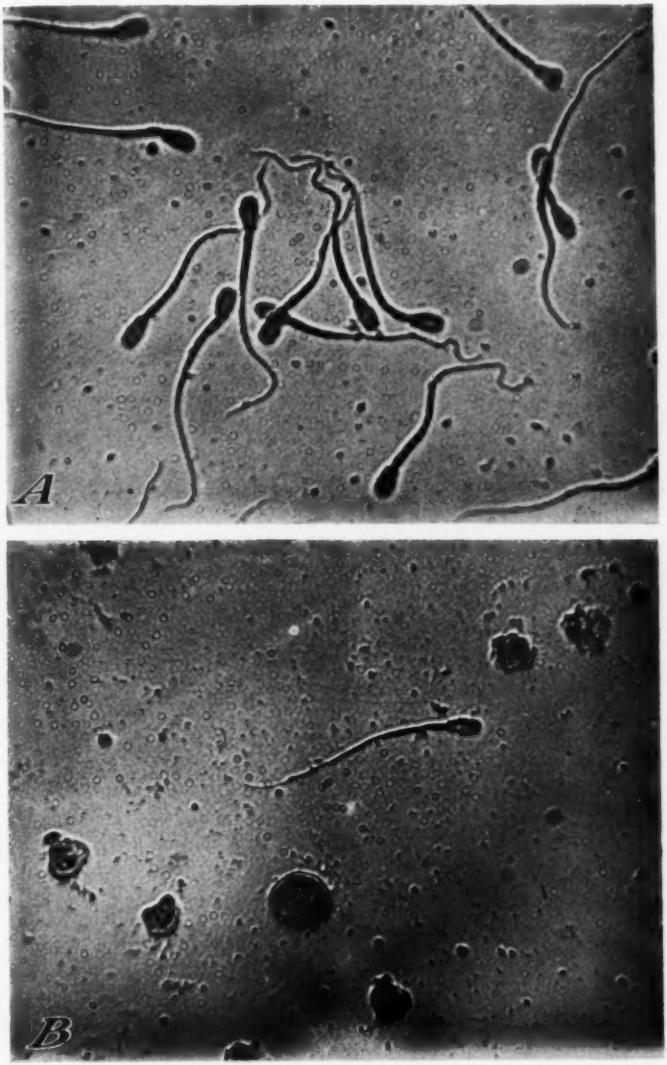

FIGURE 5.—Spermatozoa collected from bull no. 2: *A*, 513 days before grafting; *B*, 59 days after first grafting and 14 days after second grafting. × 460.

90-percent activity of spermatozoa. This activity decreased until it was 40 percent by June 1927; no activity was noted after August 1927. Morphologically the spermatozoa appeared normal, even

when there was no activity. The number varied from 3 million to 476 million in a cubic centimeter.

On December 9, 1927, two pieces of bovine testicle were attached to the roughened surface of the left trapezius thoracalis. Very little of the epididymis remained on these pieces of testicle. On December 17 the skin sutures were removed. On December 21 the lower end of the incision was enlarged and oozing pus. On Dcemeber 31 some tissue came from the wound. By January 13, 1928, the wound appeared to be healed. There was no change in this bull after the operation, and no change was noticed in his spermatozoa.

In a second transplanting on January 23, 1928, three pieces of bovine testicle were attached to the roughened surface of the right trapezius thoracalis. None of the epididymis was used. The skin sutures were removed on February 7. On February 24 a piece of decomposing testicle was protruding through the skin. It was re-

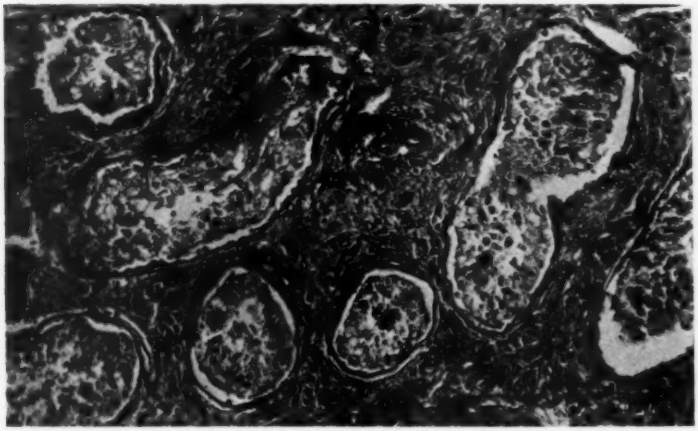

FIGURE 6.—Section from testicle of bull no. 2. × 90.

moved. The spermatozoa of this bull remained inactive, but his appetite and aggressiveness increased (fig. 5). For several years he had been unable to mount a cow because of injured rear legs and he had had to walk up on the breeding crate and maneuver into position before he could serve the cow. After the second grafting operation he mounted cows in the normal way.

On autopsy March 2, 1928, gonitis and inflammation of the hip joint with bone growth was found in both rear legs. The genital organs were normal in shape but appeared atrophied. Only scar tissue was found at the sites of grafting.

Gross sections of the testes revealed a tumor formation which histologically was found to be of the nature of a teratoma. Several of these new growths, which could be outlined fairly well in both testes on sectioning, largely replaced the testicular tissue in the areas involved. The small growths were composed of large polygonal cells, showing an alveolar arrangement in some areas, while in other parts the cells assumed more or less of an adenomatous appearance.

The tumor cells were undergoing rapid degenerative changes and on the whole stained very poorly. Outside of the growths proper, masses of the tumor cells could be seen infiltrating the interstitial

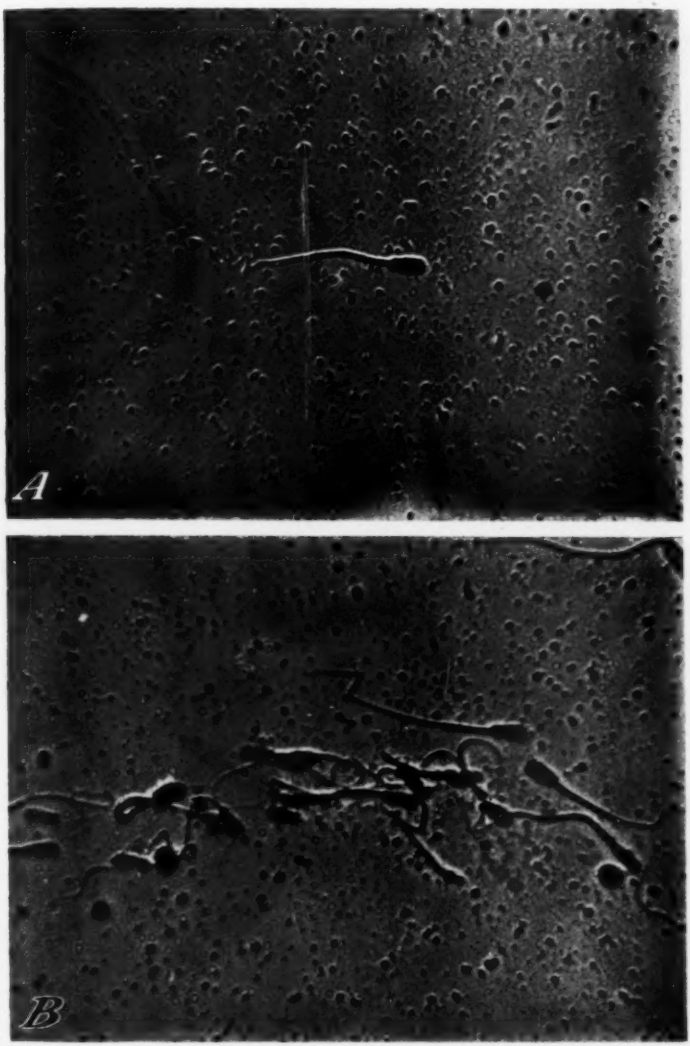

FIGURE 7.—Spermatozoa collected from bull no. 3: *A*, 9 days before grafting; *B*, 55 days after grafting. ×460.

tissue which was greatly increased throughout the testes. Many tubules had undergone complete atrophy and the remaining tubules showed varying stages of degeneration and desquamation of the

tubular epithelium. A few spermatozoa were noted in a limited number of the tubules (fig. 6).

Bull 3

Bull no. 3 was a registered Holstein born April 21, 1911. Microscopical examination of the semen of this bull revealed a 95-percent activity of spermatozoa early in 1926. The activity had decreased to 40 percent by February 1928. Morphologically the spermatozoa seemed normal until 1928, when the heads appeared narrow and long. The number of spermatozoa in a cubic centimeter varied from 1 million to 135 million.

Four pieces of pig testicle were attached to the roughened surface of the left trapezius thoracalis February 24, 1928. The skin sutures were removed March 9 and the wound healed by first intention.

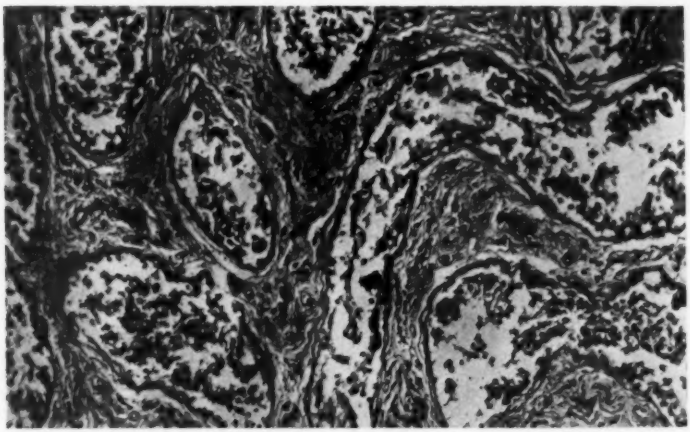

Figure 8.—Section from testicle of bull no. 3. × 90.

This bull improved in appetite and vigor. The activity of his spermatozoa increased 10 percent (fig. 7). When he was slaughtered July 9 the genital organs appeared normal. A growth was found in the thymus. Only scar tissue was found at the point of grafting.

Sections from portions of the testes showed extensive fibrous proliferation with consequent atrophy and obliteration of many of the seminiferous tubules. In those areas showing only limited fibrous changes there was degeneration and desquamation of the tubular epithelium. In some tubules there had been complete destruction of the epithelium, leaving only the basement membrane. Calcareous material was noted in a few of the tubules, and a number contained pigment deposits. Some tubules showed a limited number of spermatozoa. In the epididymis there was likewise destruction of the tubular epithelium, the lumen of the tubules being filled with the detached cells and cellular debris. There was also a noticeable increase in the interstitial tissue (fig. 8).

BULL 4

Bull no. 4 was a grade Holstein born August 18, 1921. Microscopical examination of this bull's semen showed a 90-percent activity

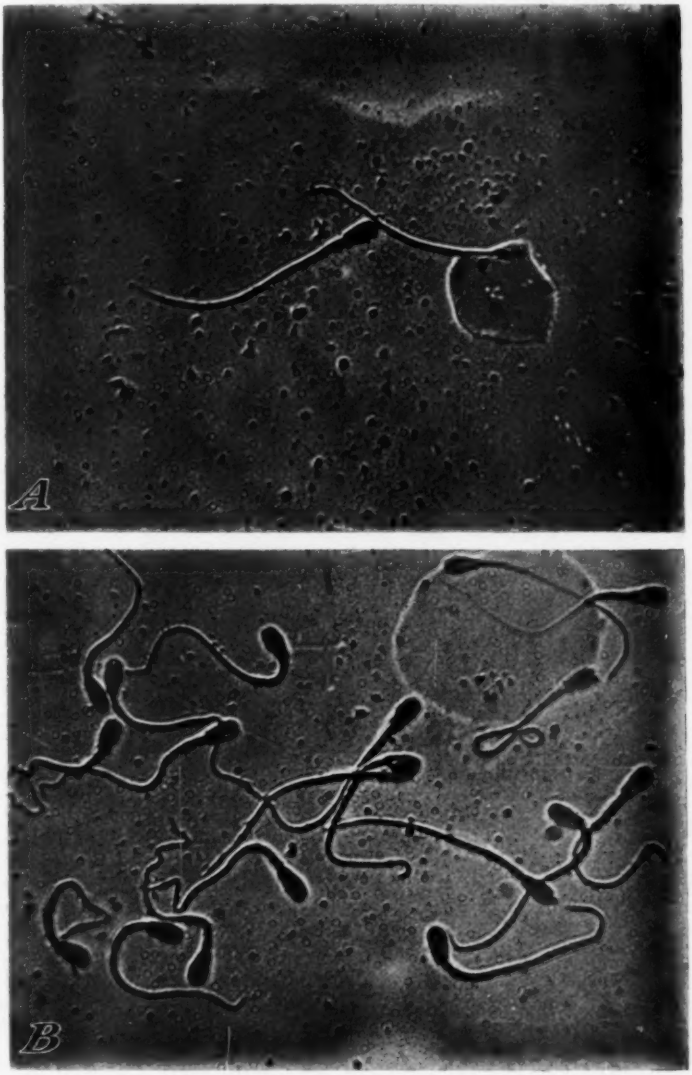

FIGURE 9.—Spermatozoa collected from bull no. 4: *A*, 41 days before grafting; *B*, 52 days after grafting. ×460.

of spermatozoa during 1926 and 1927. In January 1928 the activity was zero. Morphologically the spermatozoa appeared normal.

On February 24, 1928, four pieces of pig testicle were grafted to the roughened surface of the left trapezius thoracalis. The skin sutures were removed March 9, and the wound healed rapidly after one piece of testicle was taken out. The spermatozoa of this bull again showed an activity of 90 percent (fig. 9); table 2 shows his service record following the grafting.

This bull was slaughtered September 12, 1929, and no gross abnormalities were found. A scar was observed at the place of grafting, but no testicular tissue was found.

Sections from the testes of this animal showed a limited number of the seminiferous tubules with the epithelium intact which contained a fair number of spermatozoa. On the whole, however, there was rather advanced degeneration and desquamation of the epithelium in a very large percentage of the tubules, while in some areas there

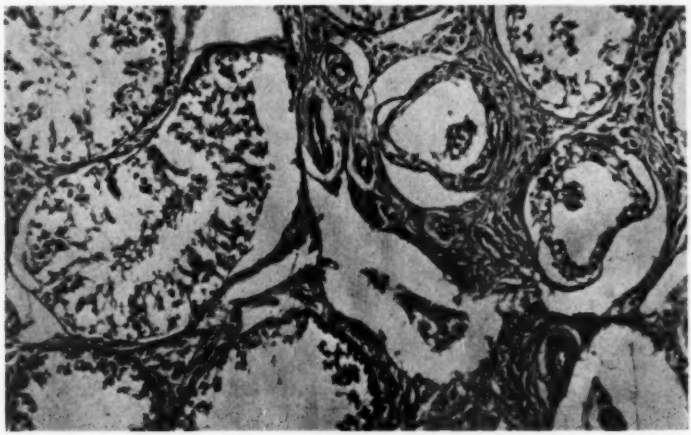

FIGURE 10.—Section from testicle of bull no. 4. × 90.

was practically complete destruction of the tubular epithelium in whole groups of tubules. There was considerable cellular infiltration with a noticeable increase in the interstitial tissue in some of the sections examined (fig. 10).

BULL 5

Bull no. 5 was a registered Holstein born June 9, 1926. He was placed in a herd when a year old, and in September 1927 it was noticed that the cows bred to him did not conceive. Spermatozoa could not be found by a careful microscopic examination of semen September 27, 1927. Examinations between September 27, 1927, and May 3, 1928, revealed pieces of heads of spermatozoa, odd-shaped heads without tails, and some tails with pieces of heads, round heads, and odd-shaped heads attached. The last examination before grafting revealed only a few pieces of spermatozoa.

On May 3, 1928, four pieces of pig testicle were attached to the roughened surface of the right trapezius thoracalis and six pieces were attached to the roughened surface of the left trapezius thora-

calis. Much fat was encountered in this animal. By May 12 the grafted area on the left side of the animal was enlarged to half the

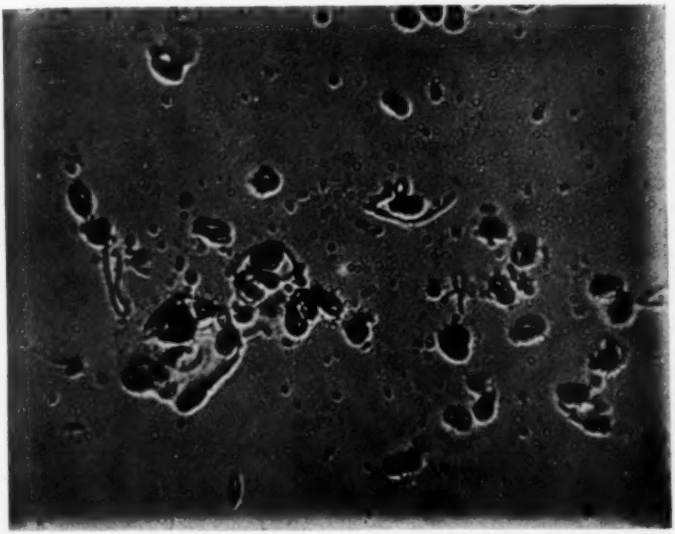

FIGURE 11. Spermatozoa from bull no. 5, collected 28 days after grafting. × 430. Spermatozoa collected before grafting were similar in appearance, but fewer in number.

size of a football. On May 14 the skin sutures on this side were removed and the enlargement was found to be a blood clot. By

FIGURE 12. Section from testicle of bull no. 5. × 90.

May 16 the incision spread open about 4 inches with the blood clot protruding. On May 26 the skin sutures on the right side were removed and the wounds then healed rapidly.

A microscopical examination of the semen of this bull on May 31, 1928, showed a few whole spermatozoa moving sluggishly; also many pieces of spermatozoa, round heads, and heads of varying shapes were found (fig. 11). Fewer pieces were observed in June, and on June 29 no spermatozoa or pieces of spermatozoa could be found.

On July 9, 1928, when this bull was slaughtered, all organs and tissues appeared normal. Only scar tissue was found at the sites of grafting.

Sections from the testes of this bull showed varying stages of degeneration of the epithelium of the seminiferous tubules. The changes were more advanced in some areas than in others. A certain percentage of the tubules showed either partial or complete desquamation of the tubular epithelium, the lumen of the tubules being filled with the desquamated cells and granular material. Only limited numbers of spermatozoa were noted even in those tubules which were practically normal in appearance. The epididymis also showed some destruction of the tubular epithelium in this case (fig. 12).

SUMMARY

Experiments were made to determine the possibility of correcting faulty reproduction in dairy cattle by the implantation of gonadial tissue.

Ovarian tissue removed from cows shortly after they were killed was grafted on 2 cows that had been affected with metritis, on 1 that had been affected with ovarian cysts, and on an apparently normal heifer. Ovarian tissue obtained from pigs in the same manner was grafted on 3 cows that had been affected with metritis, on 2 apparently normal cows, and on 2 pregnant cows.

The results show that the transplanted ovarian tissue had no appreciable effect on the oestrual cycle or on the production of ova. Apparently no damage was done to pregnant cows. A temporary change was noticed in the appearance of the vulva of one cow.

Testicular tissue obtained from a young live bull was grafted on 2 senile bulls. Testicular tissue obtained from live pigs was grafted on 2 senile bulls (one of these also had bovine testicle grafts), on 1 bull that was 6 years 6 months old and temporarily sterile, and on 1 young bull showing aspermia.

The results show that the transplanted testicular tissue had no appreciable effect on the spermatozoa of senile bulls, although it did seem to increase their appetite, body vigor, and aggressiveness. The spermatozoa of the 6½-year old bull became active and fertile following this treatment. This was not necessarily due to the transplanted tissue, however, since a change in feed and management has produced the same results in other bulls. In the young bull showing aspermia, an increase in pieces of spermatozoa was noticed, but there was not enough improvement to enable the bull to become fertile.

In both the bulls and the cows healing was much more rapid where gonadial tissue from the pig was used.

THE CYSTINE, TRYPTOPHANE, AND TYROSINE CONTENT OF THE SOYBEAN[1]

By FRANK A. CSONKA, *senior chemist*, and D. BREESE JONES, *principal chemist, Protein and Nutrition Division, Chemical and Technological Research, Bureau of Chemistry and Soils, United States Department of Agriculture*

INTRODUCTION

The importance of the quality of the protein in the diet both in human nutrition and in animal nutrition is being increasingly recognized. Although the protein content of food is usually estimated on the basis of its nitrogen percentage, the values thus obtained do not constitute fair criteria by which the protein nutritional value of the food may be determined. Neither the presence in the food of nitrogenous substances other than protein nor the quality of the protein itself is taken into consideration. It has been well established that the amino acids lysine, tryptophane, cystine, and histidine are indispensable dietary components for the normal growth and nutrition of animals. These amino acids must be supplied in the diet, chiefly through the food proteins, because the animal body cannot synthesize them.

Proteins which contain these indispensable amino acids in sufficient quantities as supplied in the daily ration are considered of good quality. Many purified proteins, mostly of vegetable origin, were prepared in this laboratory, and their content of these amino acids was quantitatively determined. When the daily ration contains a known amount of these purified protein preparations, therefore, we may calculate accurately the quantity of the indispensable amino acids present. In the past, experimental diets of such composition were used to determine the nutritional value of purified proteins. However, when the daily ration consists of a staple food like soybean (*Soja max*), for instance, such analytical data are insufficient. The quantity of the indispensable amino acids in the glycinin is known, but the percentage of glycinin in soybeans is unknown, and probably varies in different varieties. Moreover, besides glycinin there are other proteins in the soybean (*4*)[2] of which chemically very little is known. The determination of these essential amino acids in the whole seed will enable one to account for some of the failures in feeding experiments encountered by nutritional workers. The soybean is especially suitable for establishing such analytical data because one solvent (salt solution) removes from 85 to 90 percent of the nitrogen. Information of this kind will also assist the farmer in deciding which of the varieties he should select for planting in order to produce more protein and protein of better quality from the nutritional standpoint.

EXPERIMENTAL METHODS AND DATA

Soybeans[3] were ground to a meal in a mill and most of the oil was removed by extraction with ether. The defatted meal was then

[1] Received for publication Apr. 16, 1934; issued August 1934. Presented at the eighty-sixth meeting of the American Chemical Society, Chicago, Sept. 11–16, 1933.

[2] Reference is made by number (italic) to Literature Cited, p. 96.

[3] The soybeans used in this investigation were supplied by W. J. Morse, of the Bureau of Plant Industry, U.S. Department of Agriculture.

sifted in order to eliminate the greater part of the hulls. Tryptophane and tyrosine determinations were made directly on the meal. The colorimetric method of May and Rose (*6*), as modified by Csonka (*1*), was used for determining tryptophane. Tyrosine was determined by the method of Folin and Ciocalteu (*3*). It was found that cystine could not be determined satisfactorily on the meal itself, but that the Sullivan colorimetric method (*10*) as modified by Csonka (*1*) could be applied to a 10-percent sodium chloride extract of the meal. Preliminary tests showed that from 85 to 90 percent of the total nitrogen of the meal is extractable by a 10-percent sodium chloride solution. Inasmuch as the extraction residue doubtless contains some cystine, the values obtained on the extract must be considered minimal. It is believed, however, that they do give a fairly representative evaluation of the cystine content of the meal.

The sodium chloride extracts of the soybean meal used for the cystine determinations were prepared in the following manner: Samples consisting of 2.5 to 3 g of the air-dried, defatted meal were introduced into two centrifuge tubes, and to each was added from 10 to 12 cc of a 10-percent sodium chloride solution. The mixture was frequently stirred during an extraction period of half an hour. After the mixture had been centrifuged, the supernatant liquid was decanted into a 100-cc graduated cylinder. The extraction was repeated in a similar manner five times. The time for the last two extractions was extended to three-quarters of an hour. Twenty-five cubic centimeter portions of the combined extracts were used for the cystine determinations. Sufficient concentrated sulphuric acid was added to each portion to bring the concentration to 6N, and the mixture was boiled from 18 to 20 hours. Approximately 95 percent of the acidity was neutralized by adding, drop by drop, with shaking and cooling, an approximately 40-percent solution of sodium hydroxide. The hydrolysate was then transferred quantitatively to a 50-cc volumetric flask which was filled to the mark by the addition of distilled water. The solution was decolorized by shaking it gently for 3 minutes in an Erlenmeyer flask with 2 g of kaolin. Five cubic centimeters of the filtrate were used for the color development, according to the method of Sullivan (*9*).

The results given in tables 1 and 2, represent averages of duplicate analyses in which differences were seldom more than 0.03 percent in cystine, and but slightly higher in the tryptophane and tyrosine determinations.

The varieties of soybeans analyzed are listed in the tables in the order of their nutritive value as judged by their cystine and tryptophane content. In general, the nitrogen content of these varieties parallels the cystine and tryptophane content.

Because of variations in the nitrogen content of the different varieties of soybeans, table 2 was constructed to show the quantity of amino acids per gram of nitrogen in the beans.

DISCUSSION

According to Mitchell and Smuts (*7*), the question of cystine deficiency has to be considered when the protein requirement in the daily ration is supplied solely by the soybean, as the other essential amino acids are apparently present in adequate quantities. It seemed especially desirable, therefore, to obtain information on the cystine

content of the whole bean. The results of this investigation, in which the cystine determinations were made on the defatted meal, showed that the cystine percentage is not the same in all varieties of soybeans. Although it is claimed by Lugg (*5*) that cystine determination in the presence of carbohydrates (particularly cellulose) involves losses, the findings of the present investigation confirm those obtained by the authors in an earlier study of the cystine content of several varieties of soybeans (*2*), in which the cystine determinations were carried out on the glycinin preparations.

TABLE 1.—*Nitrogen and amino acid content of different varieties of soybeans grown at Arlington Farm and elsewhere under known optimum environmental conditions, expressed in percentages calculated on a moisture-free basis*

GROWN AT ARLINGTON FARM

Seed					Composition of the defatted meal			
Variety serial no. or variety	Origin	Color	Practical application	Nitrogen content	Nitrogen	Cystine	Tryptophane	Tyrosine
82284	Korea	Yellow	Tofu or bean curd.	7.41	9.87	0.476	1.17	2.61
85127	do	Green	Food	7.28	8.72	.473	1.12	2.29
85104	do	do	do	7.52	9.91	.407	1.09	2.63
80459	Japan	Yellow	Tofu or bean curd.	7.12	9.28	.396	1.11	2.44
85669	do	do	do	6.84	8.73	.389	.953	2.59
85123	Korea	Green	Food	7.39	8.96	.349	.933	3.01

GROWN UNDER OPTIMUM ENVIRONMENTAL CONDITIONS

Herman		Yellow		7.45	10.51	0.491	1.13	2.68
Mammoth Yellow		do		7.32	10.17	.334	1.02	2.49
Tokyo		Green		7.06	9.43	.338	1.00	2.45
Peking		Black		5.51	8.70	.388	.93	2.76
Illini		Yellow		5.90	8.66	.287	1.03	2.68
Chiquita		do		7.02	9.28	.324	.91	2.54

TABLE 2.—*Relationship between nitrogen content and the amino acids cystine and tryptophane of soybean meal*

Variety of soybean	Milligrams of indicated amino acid per gram of nitrogen		Variety of soybean	Milligrams of indicated amino acid per gram of nitrogen	
	Cystine	Tryptophane		Cystine	Tryptophane
Herman	46.7	107.5	Tokyo	35.9	106.0
Peking	44.6	106.9	Chiquita	46.2	90.1
Illini	33.1	119.0	Mammoth Yellow	32.8	100.3

The protein content of the soybean is influenced by climatic conditions. If a variety is capable of maturing in the northern part of the United States as well as in the southern under equal cultural conditions, the one grown in the North will have the tendency to produce a seed with higher protein content (*9, pp. 124–126*). The findings of these experiments show that the cystine content of the bean generally parallels the nitrogen content. That the cystine content varies in different varieties is clearly shown in the two sets of samples analyzed, one of which was grown at Arlington Farm and the other in localities in which the environmental conditions were known to be suited for

optimum growth and development (table 1). In view of these findings it is possible, therefore, that cystine deficiency might be encountered in feeding experiments when the lower cystine-containing varieties are fed. However, it is not fair to assume that soybean protein in all varieties is incomplete from the nutritional standpoint unless it is shown by feeding experiments that the cystine deficiency cannot be accounted for by varietal differences. Osborne and Mendel (*8*) have pointed out that cooking soybean meal in water renders it more palatable and increases the utilization of the protein by making the amino acids more available for assimilation. The analytical data here presented do not support the idea of a quantitative deficiency of cystine in soybean proteins, but they do admit the possibility of a qualitative deficiency, due to low availability of amino acids, when the raw meal is fed, particularly when soybeans containing the lower cystine values are used.

SUMMARY

The amino acids cystine, tryptophane, and tyrosine in the soybean were determined quantitatively. It was found that the percentage of these amino acids varies in different varieties. It is suggested that varieties should be selected for planting which produce more and better quality protein from the nutritional standpoint.

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